Regulation of stem cell characters in the haploid generation of the moss *Physcomitrella patens*

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. GENERAL ITRODUCTION

Land plants have a haplodiplontic life cycle, in which gametophyte and sporophyte generations alternate (Gifford and Foster, 1989). In seed plants, although multicellular bodies are formed in both gametophyte and sporophyte generations, the dominant generation is the sporophyte generation. Non-seed plants also form multicellular bodies in both gametophyte and sporophyte generations. However, both gametophyte and sporophyte generations exist independently in fern and lycophytes, and the gametophyte generation is the dominant generation in bryophytes. In addition, the closest relatives of land plants, the charophytes, have a multicellular body in the gametophyte generation and form only a zygote as a sporophytic cell. Since the morphological similarity was observed between the gametophyte and sporophyte in early fossil land plants, it is hypothesized that the developmental mechanisms in the sporophyte generation were co-opted from pre-existing mechanisms in the gametophyte generation (Kenrick and Crane, 1997; Nishiyama et al., 2003; Friedman et al., 2004). In order to test the hypothesis, it is necessary to analyze the developmental mechanisms in both gametophyte and sporophyte generations.

Shoot apical meristem (SAM) exists at the growing tips of a plant body to generate the aboveground portion in the sporophyte generation of vascular plants. The SAM can be divided into three organized zones. The central zone contains stem cells, the organizing center contains niche cells, and the peripheral zone contains cells to be differentiated. SAM is a good model to analyze molecular mechanisms for stem cell regulation in the sporophyte generation, and using *Arabidopsis thaliana* as a model

plants, various researches were extensively performed (reviewed in Sablowski, 2011; Perales and Reddy, 2012). Such a stem cell system is also a good model to compare the molecular mechanisms in both gametophyte and sporophyte generations, because stem cells also exist in the gametophyte generation of non-seed plants to generate the multicellular bodies. However, compared to a huge amount of researches for stem cells in the sporophyte generation of flowering plants, there are a few researches for stem cells in the gametophyte generation of non-seed plants. The moss *Physcomitrella patens* is a good model organism for the purpose of analyzing the molecular mechanisms for stem cell regulation in the gametophyte generation because of the following three reasons: (1) a simple stem cell system, (2) availability of various molecular biology techniques, and (3) phytohormone mediated development of the gametophyte.

(1) Simple stem cell system in *P. patens*

A single protonemal stem cell is formed at the first cell division of a spore in *P. patens*. A protonemal apical stem cell performs only apical growth and cell division to produce differentiate protonemal cells, called protonemata. These differentiated protonemata form a single side branch initial cell. Then the side branch initial cell differentiates into a protonemal apical stem cell or a gametophore apical stem cell, another stem cell in *P. patens*. A gametophore stem cell then produces a gametophore, which form a three-dimensional stem and leaf structure. In this stem cell system, a single side branch initial cell directly differentiates into two different types of stem cells. This simple stem cell system allows us to investigate developmental processes at the cellular level.

(2) Availability of various molecular biology techniques

The whole genome sequence of *P. patens* has been revealed (Rensing et al., 2008).

The transformation of *P. patens* has been established well and it is reported that the homologous recombination occurs efficiently in the genomic DNA of *P. patens* (Schaefer et al., 1991; Schaefer and Zrÿd, 1997). The gene targeting via homologous recombination, is a powerful tool to analyze the function of genes in *P. patens*. In addition, various vectors for gene targeting are available.

(3) Phytohormone mediated development of the gametophyte

The developmental processes of *P. patens* are regulated by phytohormones (e.g. auxin and cytokinin) similar to those regulating angiosperm development (Cove et al., 2006). Treatment of protonemata with exogenous cytokinin induced the formation of gametophore apical stem cells. Furthermore, some cytokinin resistance mutants showed defects in gametophore production and such defects were repaired by the addition of auxin, implying interaction between auxin and cytokinin for gametophore formation, although responsible genes were not identifies (Ashton et al. 1979).

KNOX and WUSCHEL (WUS)-CLAVATA3 (CLV3) pathways are two main pathways to regulate stem cells in SAM of flowering plants (Endrizzi et al., 1996; Mayer et al., 1998; Schoof et al., 2000). SHOOT MERISTEMLESS (STM), one of Class 1 *KNOX* genes, functions in establishment and maintenance of a SAM, and loss of

function of these genes fails to form and maintain a SAM. *WUS* gene is expressed in the organizing center and necessary to maintain stem cells in the central zone via positive regulation of *CLV3* gene, which is expressed in stem cells. *CLV3* gene negatively regulates the expression of *WUS* gene and this negative feedback loop between *WUS* and *CLV3* genes is necessary to maintain a stem cell population in a SAM. To analyze the function of such genes in stem cells in the gametophyte generation is the first step to assess the "co-option" hypothesis.

Previous studies showed orthologs of class 1 *KNOX* and paralogs of *WUS* genes exist in *P. patens* (Deveaux et al., 2008; Sakakibara et al., 2008). It is shown that orthologs of class1 *KNOX* genes do not function in the gametophyte stem cells and they function in development of a sporophyte in *P. patens* (Sakakibara et al., 2008). There is no report for the function of paralogs of *WUS* genes in *P. patens*. Therefore, I generated disruption lines of *WOX* genes in *P. patens*. These disruption lines showed normal growth and no morphological difference compared with wild type in the gametophyte generation (data not shown). Recently, it was shown that loss-of-function of *WOX* genes caused defects in regeneration from leaf cells and sporophyte development in *P. patens* (Sakakibara et al., submitted). These studies suggest the possibility that there are different molecular mechanisms for stem cell regulation between gametophyte and sporophyte generations. However, it is still difficult to deny the "co-option" hypothesis because there is very few information for genes involved in stem cell regulation in the gametophyte generation. Therefore, it is necessary to find genes involved in stem cell regulation in the gametophyte generation and analyze the functions of those genes in the

gametophyte generation of non-seed plants and their orthologs in the sporophyte generation of flowering plants in order to test the “co-option” hypothesis.

2. AP2-TYPE TRANSCRIPTION FACTORS DETERMINE STEM CELL IDENTITY IN THE MOSS *PHYSCOMITRELLA PATENS*

2.1 Introduction

Stem cells are characterized by their ability to self-renew and give rise to differentiated cells (Lajtha, 1979). Land plants have a haplodiplontic life cycle, in which gametophyte and sporophyte generations alternate (Prigge and Bezanilla, 2010). Stem cells are formed only during sporophyte development in flowering plants, whereas mosses, a basal group of land plants, form stem cells in both the sporophyte and gametophyte generations (Sakakibara et al., 2008; Kofuji et al., 2009). Since the closest relatives of land plants, the charophytes, have a haplontic life cycle and retain stem cells only in the gametophyte generation, it is hypothesized that the molecular mechanisms underlying stem cell regulation in sporophytes were co-opted from pre-existing mechanisms in gametophytes (Kenrick and Crane, 1997; Nishiyama et al., 2003; Friedman et al., 2004). However, previous studies in the moss *Physcomitrella patens* showed that class 1 KNOX genes, which regulate the initiation and maintenance of stem cells in flowering plant shoot meristems, did not function in the haploid stem cells of the moss (Sakakibara et al., 2008). In contrast to the advancements made in our understanding of the molecular mechanisms underlying stem cell regulation in the sporophyte generation of flowering plants, only a few genes responsible for gametophyte stem cell formation have been reported (Jang et al., 2011). It is important to identify the genes that regulate gametophyte stem cell formation in order to elucidate the general principles of stem cell

formation in plants.

The moss *Physcomitrella patens* is a useful model organism for investigating the regulation of stem cells in the gametophyte generation. Two types of bodies are formed in this moss: filamentous bodies, called protonemata, and gametophores, which consist of stems and leaves. The two main types of protonemata, i.e., chloronemata and caulonemata, can be distinguished by their chloroplast morphology, cell length, tip growth rate, and cross wall orientation (Cove et al., 2006; Prigge and Bezanilla, 2010). A primary chloronema apical cell is initiated during spore germination and exhibits tip growth. The primary chloronema apical cell is a stem cell, which is maintained by self-renewal and produces chloronema cells by continuous cell divisions. Primary chloronema apical cells develop into caulonema apical cells, which produce caulonema cells. Caulonema cells form side branch initial cells of which approximately 87% becomes secondary chloronema apical cells, 5% secondary caulonema apical cells, 5% gametophore apical cells, and 3% undivided cells under regular culture conditions (Fig. 1) (Cove and Knight, 1993). Gametophore apical cells divide to form gametophores, which develop stems and leaves, and later archegonia and antheridia, which facilitate sexual reproduction. Apical cell formation is regulated by two phytohormones, cytokinin and auxin. The exogenous application of cytokinin enhances the formation of gametophore apical cells (Ashton et al., 1979; Cove et al., 2006). In addition, the exogenous application of auxin to cytokinin-resistant mutants restored gametophore formation. This suggested that these mutants had some defects in auxin biosynthesis and auxin was necessary for cytokinin signaling during gametophore apical cell

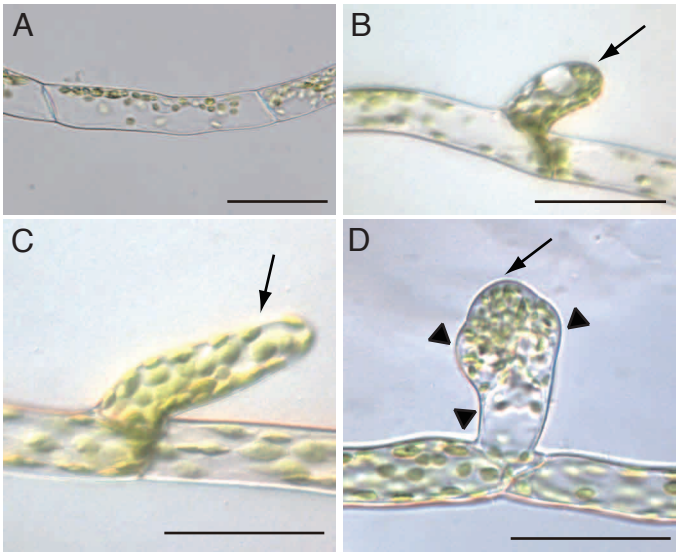

Figure 1. Formation of secondary protonema apical cells and gametophore apical cell from caulonema cells.

(A) Caulonema cells. (B) A side branch initial cell (indicated by an arrow) is formed from a caulonema cell. (C) Approximately 92% of side branch initial cells are fated to beome a secondary protonema apical cell (indicated by an arrow). (D) Approximately 5% of side branch initial cells are fated to become a gametophore apical cell (arrow) and divide to form gametophore cells (arrowheads).

Scale bars: 50 μm.

formation (Ashton et al., 1979). Furthermore, it was shown that auxin and cytokinin signaling act in the same pathway (Prigge et al., 2010).

The coordination of auxin and cytokinin signaling in the regulation of stem cell formation in flowering plants is mediated by several transcription factors, such as *WUSCHEL*, *AUXIN RESPONSE FACTOR*s (*ARF*s), and *ARABIDOPSIS RESPONSE REGULATOR*s (*ARR*s) (Zhao et al., 2010). However, no transcription factors have been shown to regulate secondary protonema apical cell and gametophore apical cell formation via phytohormone signaling.

The AP2-type transcription factors, which are characterized by the AP2/ERF DNA-binding domain, form a plant-specific protein family (Riechmann and Meyerowitz, 1998). The AINTEGUMENTA (ANT) subfamily consists of eight genes: *ANT*, *AINTEGUMENTA-LIKE* (*AIL*) *1*, *PLETHORA* (*PLT) 5*/*AIL5*, *PLT1*, *PLT2*, *PLT3*/*AIL6*, *PLT7*/*AIL7* and *BABY BOOM* (*BBM*) that are involved in the development of flowering plants (Aida et al., 2004; Nole-Wilson et al., 2005; Prasad et al., 2011). In *A. thaliana*, *PLT* genes are required for stem cell niche formation in root apical meristems, and loss-of-function of these genes causes a defect in stem cell maintenance (Aida et al., 2004). A gradient of PLT proteins controls stem cell programming, mitotic activity, and cell differentiation (Galinha et al., 2007). *ANT*, another gene of this subfamily in *A. thaliana*, regulates cell proliferation and organ growth during auxin signaling (Elliott et al., 1996; Mizukami and Fischer, 2000; Hu et al., 2003). *BBM* is thought to be involved in embryogenesis, because its over-expression induces somatic embryogenesis in *A. thaliana* (Boutilier et al., 2002). However, the functions of genes in

this subfamily have not been characterized in non-flowering plants.

Here, I show that four *P. patens* genes orthologous to *ANT*, *PLT*, and *BBM* are indispensable for the formation of gametophore apical cells. I also reveal that these genes are transcriptionally regulated by auxin, and are required for the cytokinin-dependent induction of gametophore apical cells. I conclude that the AP2-type genes function as a molecular switch to promote the development of different types of stem cells in the *P. patens* gametophyte generation.

2.2 Materials and Methods

2.2.1 Plant material and culture conditions

Physcomitrella patens Bruch and Schimp. subsp. *patens* collected in Gransden Woods (Ashton and Cove, 1977) was used as the wild-type line and was propagated on BCDAT medium (Nishiyama et al., 2000) at 25°C under continuous light. For heat shock induction, protonemata were cultured at 25°C and kept at 38°C for 1 hour of every 12 hours. To analyze the effect of auxin and cytokinin on the activity of *APB* genes, protonemata were cultured on BCDAT medium for four or five days under continuous light and then transferred to BCDATG medium and cultivated for seven days under unilateral red light. After cultivation, the moss plants were transferred to a two-fold dilution of liquid BCD medium (Nishiyama et al., 2000) with various concentrations of benzylaminopurine (BAP) and naphthalenacetic acid (NAA) and cultivated for two days under white light.

2.2.2 Phylogenetic analysis

I used a data set of AP2-type transcription factor homologs obtained using the amino acid sequence of APB1 protein as a query for a BLASTP search (Altschul et al., 1997) against the non-redundant protein data set from the National Center for Biotechnology Information. Deduced amino acid sequences were aligned with clustalW (Thompson et al., 1994) in a MEGA5 package (Tamura et al., 2011) and then revised manually. MOLPHY version 2.3b3 program package (Adachi and Hasegawa, 1996) was used for construction of phylogenetic trees. After exclusion of short or redundant sequences, 15 representative land plants (*Physcomitrella patens*, *Selaginella moellendorffii*, *Ceratopteris thalictroides*, *Gnetum parvifolium*, *Cycas revoluta*, *Ginkgo biloba*, *Pinus thunbergii*, *Picea abies*, *Vitis vinifera*, *Glycine max*, *Populus trichocarpa*, *Arabidopsis thaliana*, *Zea mays*, and *Oryza sativa*) were selected and 92 amino acid sequences were used to calculate maximum-likelihood (ML) distances for 107 genes using the JTT model (Jones et al., 1992) with a ProtML program and to construct a neighbor-joining (NJ) tree (Saitou and Nei, 1987) with a NJdist program. The ML tree was searched by local rearrangement using this NJ tree as a start tree under the JTT model with a ProtML program. Local bootstrap probability was estimated using the resampling-of-estimated-log-likelihood (RELL) method (Kishino et al., 1990; Hasegawa and Kishino, 1994).

2.2.3 Construction of plasmids for gene targeting

A schematic diagram of the disruption construct and the primer sequences are given in Fig. 2 and Table 1, respectively. To delete *APB1*, a genomic DNA fragment of *APB1*

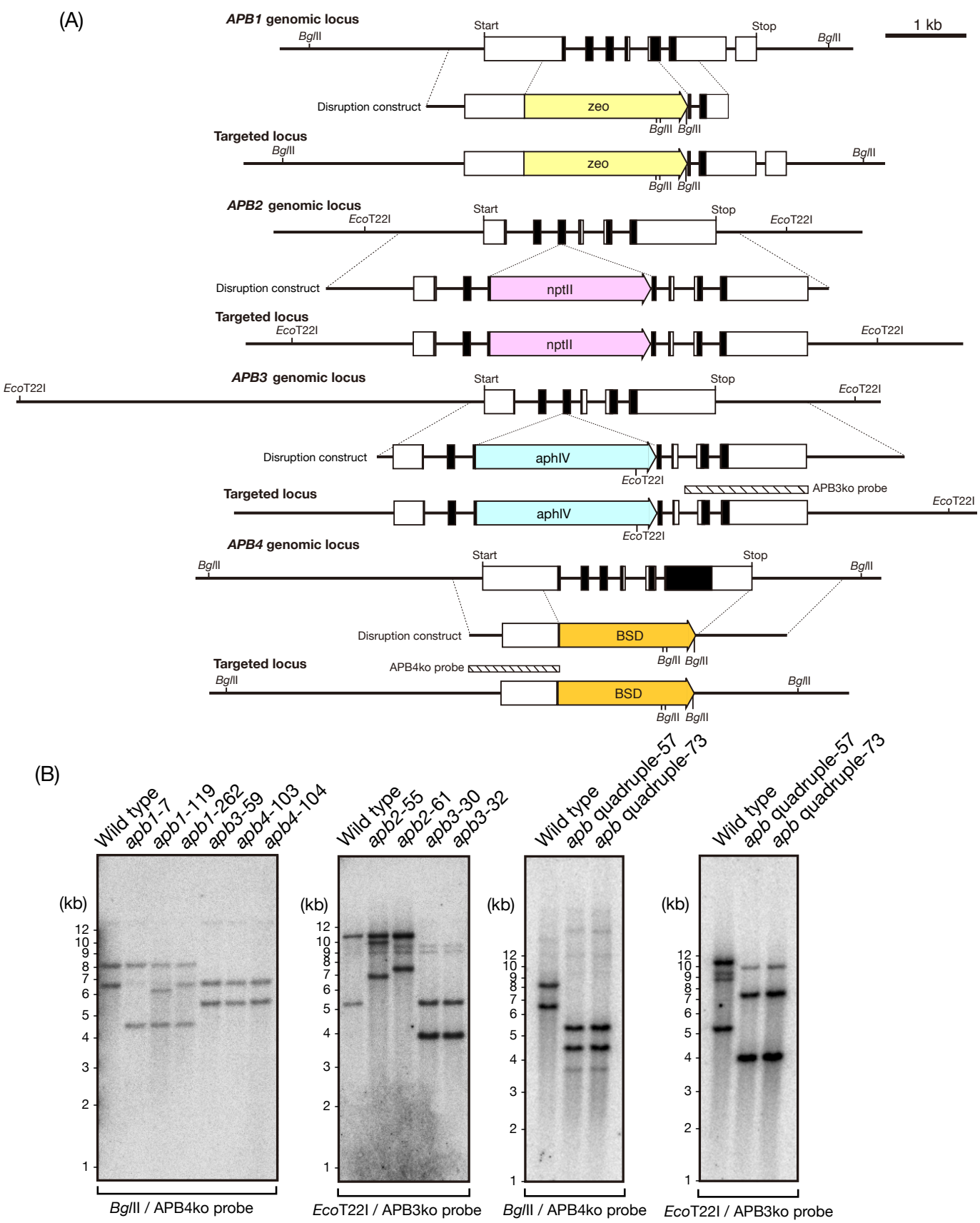

Figure 2. Generation of *APB* disruption lines.

(A) Schematic diagrams of the disruption of the *APB1*, *APB2*, *APB3*, and *APB4* loci. Boxes represent *APB1*, *APB2*, *APB3*, and *APB4* coding regions and those filled in black indicate AP2 domains. Probes used in (B) are indicated by hatched boxes. APB4ko and APB3ko probes were also used as APB1ko and APB2ko probes, respectively, because of sequence identity between the genes. Yellow, pink, pale blue, and orange arrows indicate the bleomycin expression cassette (ze0; p35S-Zeo [EF451822]) (Hiwatashi et al., 2008), the neomycin phosphotransferase expression cassette (aphIV; pTN86 [AB267705]) (Hiwatashi et al., 2008), and the blasticidin S deaminase expression cassette (BSD; p35S-lox-BSD [AB537973]), respectively. (B) Southen hybridization analysis of the deletion lines. Genomic DNA of the wild type and disruption lines was digested with BglII and hybridized with the APB4ko probe (first and third panels), and digested with EcoT22I and hybridized with the APB3ko probe (second and fourth panels).

Table 1. Primers used in this study

Primer	Sequence (5' to 3')
FPpANT1GUS+KpnI	CGGGTACCCGTATGAGGCGGAGCTGGAG
FPpANT11+H	CCCAAGCTTAGCGATGCTATCTGCTCCTC
FPpANT119+X	ATCTAGATTTGCAGAGGAGGAGGGGGTTTG
FPpANT126GUS_Xba	GCTCTAGAAGATCGGTAATGGTGTAAAG
PNT3+5-1GUS	CCTCTAGAGTTTGCCGAACACGAGCTCTC
PpA2ta3	AGGTGGGTTCTAGCATTAGCGCAAC
PpANT2+H	CCCAAGCTTTCGTTCCATGAGTG
PpANT2-M	ATGTGGCTAAGACATCAAGC
PpANT2-3-1-all	CATCGATCAATTGTACATGCCGCCAATGT
PpANT2-3-1-all-nest	ATCAATTGTACATGCCGCCAATGTTAAGGT
PpANT2-5-1-nest-all	AGTGACGCGGAAGCCCTATCTGA
PpANT3-3-1	GAGGTTAAATAACTGAATCAGTTCCACTCATG
PpANT3-5-1-nest	CATCGTCGTTTCGAATCGCATACG
PpANT3+HindIII	CCCAAGCTTTCGTTCCATGACT
PpANT4F1SalI	ATGCGTCGACTCGTTCATTTGCATCCAATC
PpANT4F2XbaI	AGGTCTAGAGTAGGAATGGGCGGTAGT
PpANT4R2SacII	GTCCCGCGGATTGGCCAACATTCTTGCTC
PpANT4spF1	CCCTGCGCACTTAAGCTTCCTCGGCATAAT
PpAP2-3	GCTGGAGGAGATGAAGAACATGTCT

PpAP2-3-5-2	CTATTAACAACTGCGGATTACCCCA
PpAPB1-ctr-3F2	GATTTGAGTAATTGACATTGGTTGT
PpAPB1-ctr-3R2	CAACAAAAAGGTGTTGCTGCCTTAG
PpAPB1GSP-F1	CCATCCACGCGGTTGATAGT
PpAPB1GSP-R1	TCACAGGATCACGAAGGACAAA
PpAPB1-ox-F1	CACCATGGCACAAATGCTAACGTCTGCGT
PpAPB1-ox-R5	TTCGTTCCACGAGTGCGCAAAAATG
PpAPB1&4-ctr-5F1	GAGGAGGGGGTTTGTATGTGTTGTT
PpAPB2GSP-F1	CGGTCCGCGGGAAAG
PpAPB2GSP-R1	TGGGACTGGGAACTCGTCAT
PpAPB3GSP-F1	GGCGAATTGTCGGCATCT
PpAPB3GSP-R1	TCTGCGCCTGACCTGAGTACT
PpAPB3-ox-R4	CCATTCGTTCCATGACTGCGTAAAA
PpAPB4-ctr-3F2	CACTCGAGTAATTTAGGTATAATAT
PpAPB4-ctr-3R2	TCAAAAGAAGATAATATGCAACCTCCA
PpAPB4GSP-F3	CGTGCGCTAGTCTGTGCTAGTG
PpAPB4GSP-R3	CCACCTGGATTGGATGCAA
PpTUA1F	CGTAGGAGGGACCAGTTTGG
PpTUA1R	TGCATTCATCCCCGAGTCA
PTA1-3’f1	GTATACAAACCTGTGGACCGCCACGATGCGGT
PTA1-3’r1	TTATTCTCTCTTCGATGGCATTTAG

RPpANT1GUS+HindIII	CCAAGCTTTTCGTTCCACGAGTGCGCAA
RPpANT121GUS_Not	ATAAGAATGCGGCCGCTGAAAGCCAAACAGAGCACAA
RPpANT12+E	CCGGAATTCTCAATCCATAGTCCTCGCTC
RPpANT15+S	TCCCCGCGGTGCCACTCCATCAGGTTTTTC
SSP-PIG1bRr1	TAAGATTCTATGCACGGATAGCAAC
T7	TAATACGACTCACTATAGGG
Xb-PIGRf1	GCTCTAGAAAACATGAATAACCAAATTAAAATATTAATAA TTC

was amplified using the FPpANT11+H and RPpANT12+E primers. The amplified fragment was digested with *Hin*dIII and *Eco*RI, and cloned into the *Hin*dIII-*Eco*RI site of the p35S-Zeo plasmid (EF451822), which contains the bleomycin expression cassette (Hiwatashi et al., 2008). This plasmid was named pAPB1-dis-5. A genomic DNA fragment was amplified with the FPpANT119+X and RPpANT15+S primers. The amplified fragment was digested with *Xba*I and *Sac*II and inserted into the *Xba*I-*Sac*II site of pAPB1-dis-5, to generate the pAPB1-KO construct. The pAPB4-KO construct was made in the same way using the PpANT4F1SaII, RPpANT12+E, PpANT4F2XbaI, and PpANT4R2SacII primers and the p35S-loxP-BSD plasmid (AB537973), which contains the CaMV 35S promoter, blasticidin S deaminase gene, and CaMV polyadenylation signal. For gene targeting, DNA fragments amplified with the FPpANT11+H and RPpANT15+S primers using pAPB1-KO as template and those with the PpANT4F1SalI and PpANT4R2SacII primers using pAPB4-KO as template were used.

For construction of pAPB2-KO and pAPB3-KO plasmids, *APB2* and *APB3* genomic DNA amplified using the PpANT2-3-1-all-nest and PpANT2-5-1-nest-all primers, and the PpANT3-5-1 nest and PpANT3-3-1 primers, were cloned into the pGEM-T vector (Promega, Madison, WI, USA), thereby generating pPpANT2g and pPpANT3g plasmids, respectively. A DNA fragment conveying the neomycin phosphotransferase II expression cassette was excised from the pGFPmutNPTII plasmid (Hiwatashi et al., 2008) with *Eco*RI and *Sph*I, blunt-ended, and inserted into a blunt-ended *Aor*51HI site of the pPpANT2g plasmid. The recombinant plasmid was

designated as pAPB2-KO. The fragment was amplified with PpANT2-3-1-all-nest and PpANT2-5-1-nest-all primers using pAPB2-KO as template and used for gene targeting. To make the pAPB3-KO plasmid, a DNA fragment conveying the hygromycin phosphotransferase expression cassette (aphIV; pTN86 [AB267705]) was excised from pHTS14 (Tanahashi et al., 2005) with *Xba*I and *Hin*dIII, blunt-ended, and cloned into the blunt-ended *Bst*1107I site of the pPpANT3g plasmid. The fragment was amplified with the PpANT3-5-1-nest and PpANT3-3-1 primers using pAPB3-KO as template and used for gene targeting.

Schematic diagrams of a reporter knock-in construct are shown in Fig. 3. To generate *APB1*-Citrine and *APB4*-Citrine lines, an *APB1* genomic DNA fragment just prior to the stop codon was PCR-amplified with the PpAPB1&4-ctr-5F1 and PpAPB1-ox-R5 primers, and cloned into the *Eco*RV site of pCTRN-NPTII 2, thereby creating an in-frame fusion of the *APB1* or *APB4* coding sequence with the *Citrine* yellow fluorescent protein gene (Heikal et al., 2000) to produce pAPB1&4Citrine-5. A genomic fragment downstream of the *APB1* stop codon was amplified with the PpAPB1-ctr-3F2 and PpAPB1-ctr-3R2 primers, and cloned into the *Sma*I site of pAPB1&4Citrine-5 to generate pAPB1Citrine. A genomic fragment downstream of the *APB4* stop codon was amplified with the PpAPB4-ctr-3F2 and PpAPB4-ctr-3R2 primers, and cloned into the *Sma*I site of pAPB1&4Citrine-5 to generate pAPB4Citrine. pAPB1Citrine and pAPB4Citrine were digested with *Hin*dIII and *Xba*I for gene targeting. To fuse the *uidA* gene, which encodes ß-glucuronidase (GUS) (Jefferson, 1987), with *APB2* and *APB3*, the genomic DNA fragments of *APB2* and *APB3* were

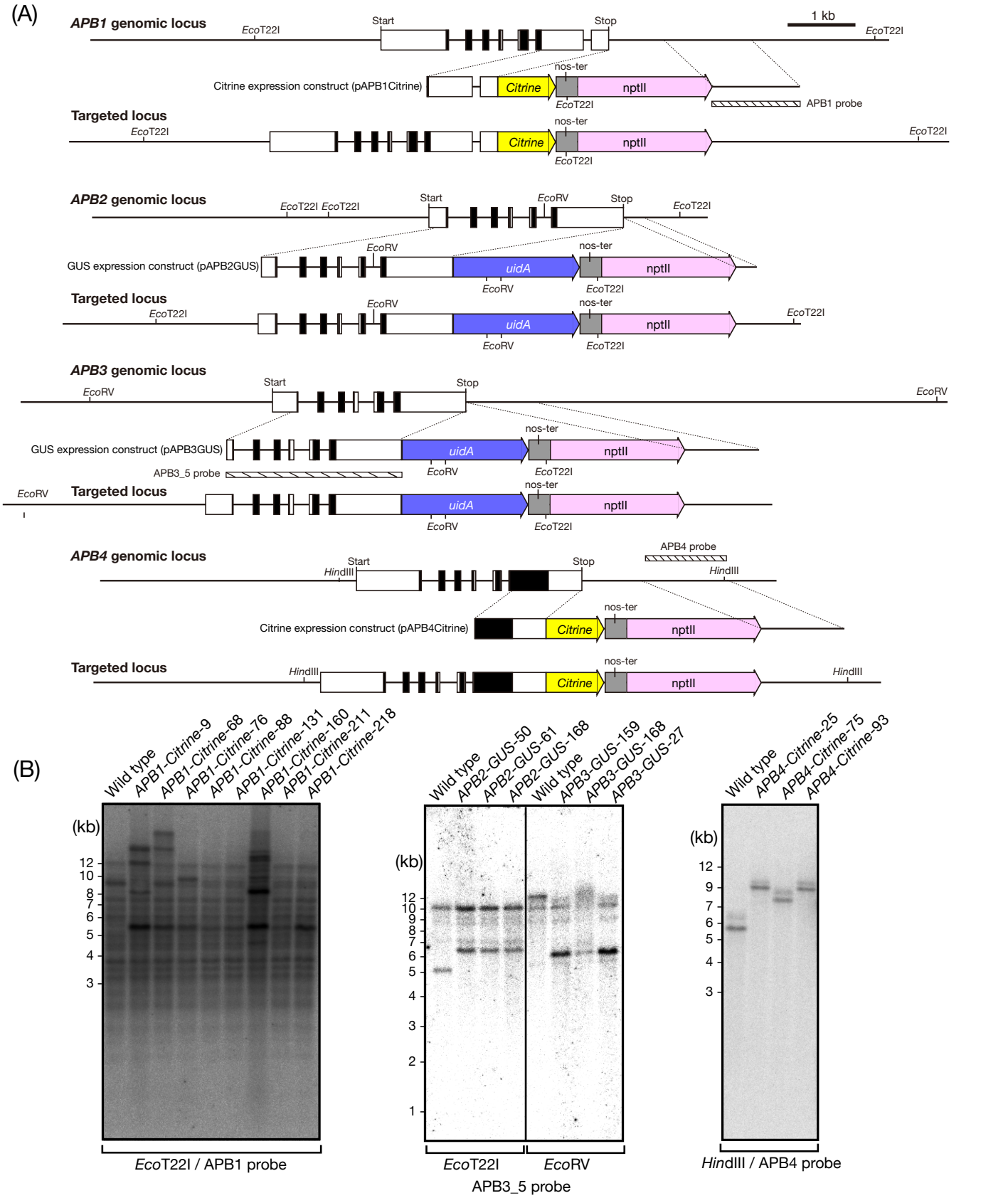

Figure 3. Generation of the *APB1-Citrine*, *APB2-GUS*, *APB3-GUS*, and *APB4-Citrine* lines.
(A) Schematic diagrams of the *Citrine* or *uidA* insertion into the *APB1*, *APB2*, *APB3*, or *APB4* locus. Boxes represent *APB1*, *APB2*, *APB3*, and *APB4* coding regions and those in black designate AP2 domains. Probes used in (B) are indicated by hatched boxes. Yellow and blue arrows denote the *Citrine* and *uidA* genes,respectively. Gray boxes and pink arrows indicate the nos terminator (nos-ter; Nishiyama et al., 2000), respectively.
(B) Southern hybridization analysis of the targeted lines. Genomic DNA of wild-type and *APB1-Citrine* lines was digested with *Eco*T22I (left panel). Genomic DNA of wild-type, *APB2-GUS*, and *APB3-GUS* lines was digested with *Eco*T22I and *Eco*RV, respectively (middle panel). Genomic DNA of wild-type and *APB4-Citrine* lines was digested with *Hin*dIII (right panel). The faint signals in the middle panel detected using the APB3-5 probe are *APB* genes that cross-hybridized with the conserved AP2 domain present in the probe.

respectively amplified with the PpANT2+H and PpANT2-M primers, and the PpAP2-3-5-2 and PpANT3+HindIII primers, using pPpANT2g and pPpANT3g plasmids as templates, and digested with *Hin*dIII. These digested fragments were cloned into the *Eco*RV-*Hin*dIII site of the pGUS-NPTII-2 plasmid (Sakakibara et al., 2003) to create an in-frame fusion of each *APB2* and *APB3* coding sequence with *uidA* and to produce pAPB2-GUS-5 and pAPB3-GUS-5. A genomic DNA fragment downstream of the *APB2* stop codon was amplified by TAIL-PCR (Liu and Whittier, 1995), cloned into pGEM-T (Promega), and named pPpANT2-tail. The DNA fragment was amplified with the T7 and PpA2ta3 primers using pPpANT2-tail as template, and cloned into the blunted *Bam*HI site of pAPB2-GUS-5 to generate pAPB2-GUS. pPpANT3g was digested with *Pst*I and the DNA fragment was blunt-ended and inserted into the blunt-ended *Bam*HI site of pAPB3-GUS-5 to make pAPB3-GUS. The generated constructs were digested with suitable restriction enzymes for gene targeting.

To construct *HSP*:*APB4* lines and *HSP*:*Cerulean-APB4* lines (Fig. 4), cDNA was synthesized using total RNA extracted from protonemata of the *apb1apb2apb3*-56 line as template using Superscript III (Invitrogen, Carlsbad, CA, USA). *APB4* cDNA was amplified using the cDNA as template and the PpANT4spF1 and PpANT2-3-1-all primers. The amplified fragment was cloned into the pCR-Blunt II-TOPO vector (Invitrogen) and named APB4-cDNA plasmid. A PCR fragment amplified using the APB4-cDNA plasmid as template with APB1-ox-F1 and APB1-ox-R5 primers was cloned into the pENTR/D-TOPO vector (Invitrogen). This plasmid was named pAPB4-entry. The pHSP-APB4 plasmid was constructed using the LR clonase reaction

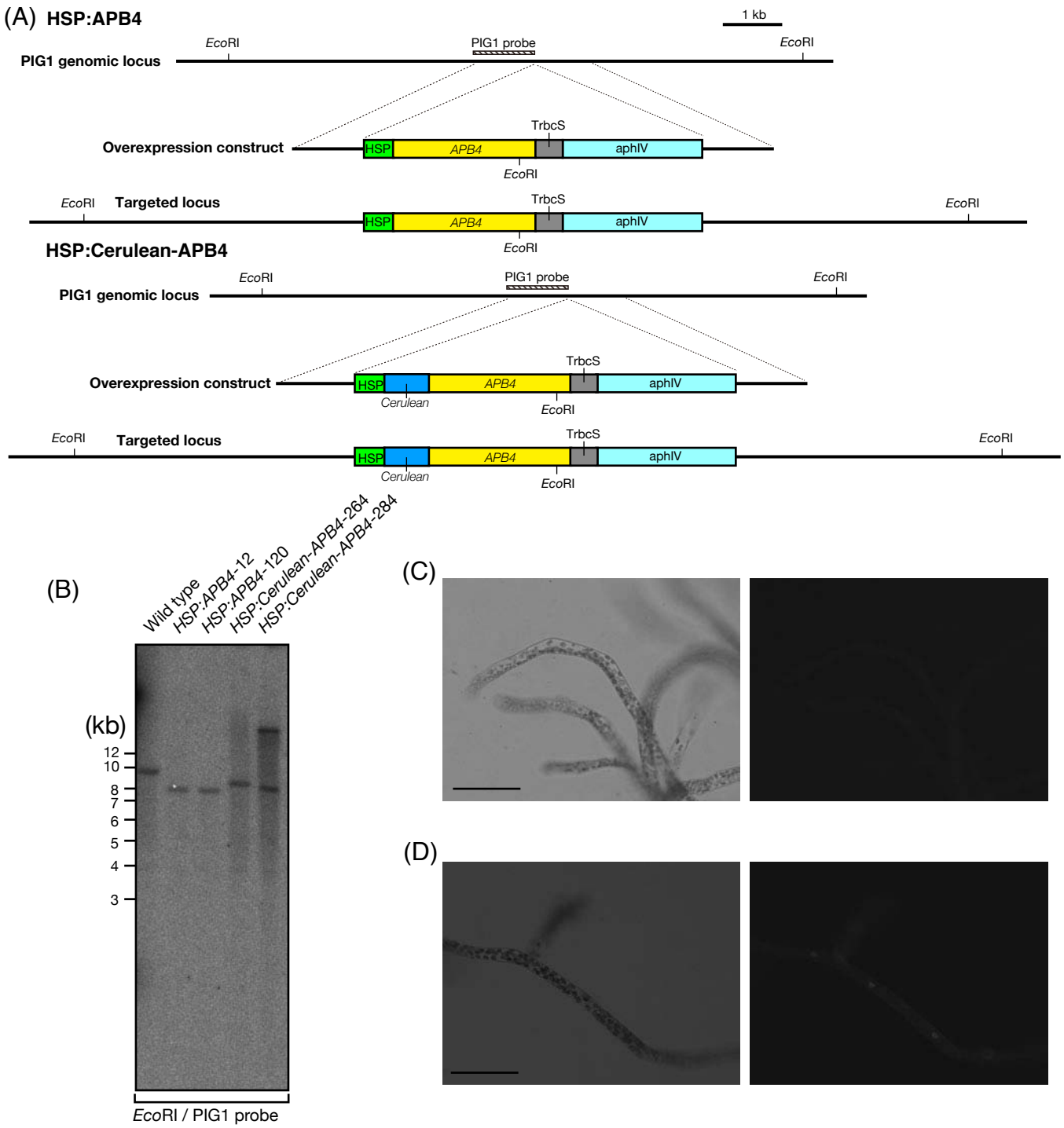

Figure 4. Construction of *HSP:APB4* and *HSP:Cerulean-APB4* lines.
(A) Schematic diagrams of targeting the PIG1 genomic locus, which is used as a potential neutral insertion site (Okano et al., 2009). Green boxes indicate a soybean *Gmhsp17.3B* (Saidi et al., 2005). Yellow boxes represent a coding region from the putative start codon to the stop codon of *APB4* cDNA. Blue boxes indicate a *Cerulean* cyan fluorescent protein gene (Rizzo et al., 2004). Probes used in (B) are indicated by hatched boxes. Gray boxes and pale blue boxes indicate a pea *rbcS* terminator (TrbcS) and the hygromycin phosphotransferase expression cassette (Hiwatashi et al., 2008), respectively. (B) Southern hybridization analysis of targeted lines. Genomic DNA of the wild-type and, *HSP:APB4*, and *HSP:Cerulean-APB4* lines was digested with *Eco*RI. (C,D) The Cerulean signal of the *HSP:Cerulean-APB4-284* line was detected in all caulonema cells after incubation at 38ºC for a hour (D), but not after incubation at 25ºC (C). Scale bars: 100 µm.

between the pAPB4-entry and pPIG1HG (AB472844) vectors to insert the putative neutral site PIG1 (Okano et al. 2009). The pHSP-Cerulean-APB4 plasmid was constructed using the LR clonase reaction between pAPB4-entry and pPIG1HCG (AB472845). Each plasmid was linearized with *Pme*I and used for gene targeting.

To construct *EF1-α:APB4* lines (Fig. 5), the pEF1-APB4-plasmid containing a *PpEF1-α* promoter region (Scaffold_7: 2739557-2740907) was constructed using the LR clonase reaction between the pAPB4-entry and pT1OG vectors to replace the insert DNA fragment to the scaffold_310: 80872-81503 (*P. patens* targeting site 1 [PTA1]) of the version 1.1 genome (http://genome.jgi-psf.org/Phypa11/Phypa11.home.html). The plasmid was linearized with *Pme*I and used for gene targeting.

2.2.4 Transformation

Polyethylene glycol-mediated transformation was performed as described previously (Nishiyama et al., 2000). Two double disruption lines of *APB2* and *APB3* (*apb2apb3*-60 and -97) were generated by inserting *APB2* into the background of the *APB3* disruption (*apb3*-30). Two triple disruption lines of *APB1*, *APB2*, and *APB3* (*apb1apb2apb3*-3 and -56) were generated by the deletion of *APB1* in the background of the double disruption line of *APB2* and *APB3* (*apb2apb3*-111). Two quadruple disruption lines (*apb1apb2apb3apb4*-57 and *apb1apb2apb3apb4*-73) were generated by the deletion of *APB4* in the background of the triple disruption lines, *apb1apb2apb3*-56 and -3, respectively. Correct gene targeting was confirmed by southern hybridization.

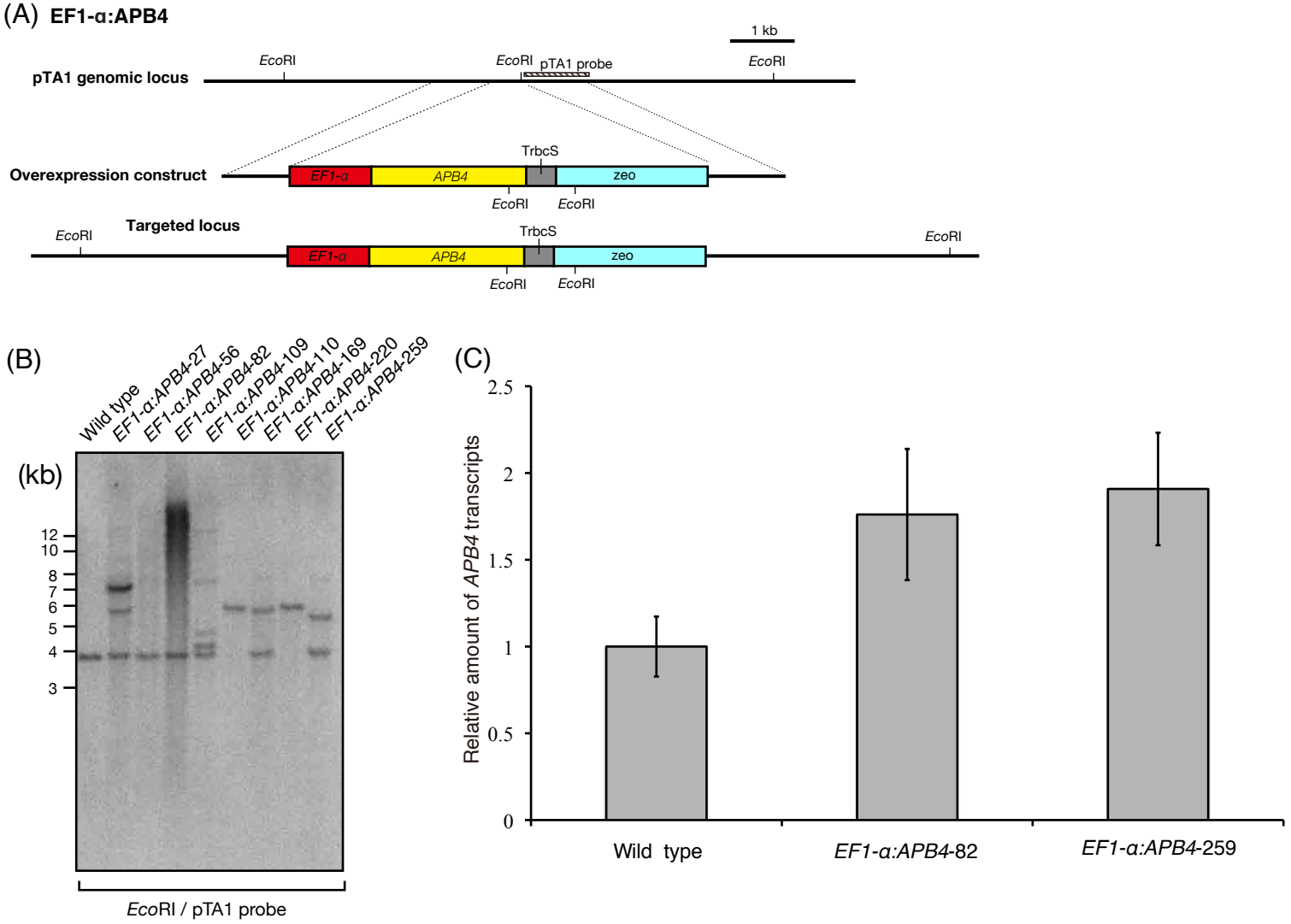

Figure 5. Generation of *EF1-α:APB4* lines.
(A) Schematic diagrams of targeting the pTA1 genomic locus. Red indicate a *EF1-α* promoter of *Physcomitrella patens*. Yellow boxes represent a coding region from the putative start codon to the stop codon of *APB4* cDNA. A probe used in (B) is indicated by a hatched box. Gray boxes and pale blue boxes indicate a pea *rbcS* terminator (TrbcS) and the bleomycin expression cassette (zeo; p35S-Zeo [EF451822]), respectively. (B) Southern hybridization analysis of targeted lines. Genomic DNA of the wild-type and *EF1-α:APB4* lines was digested with *Eco*RI. (C) Quantitative real-time RT-PCR analysis of *APB4* transcripts in transgenic lines. Protonemata were cultured on BCDAT medium for 7 days and collected. Error bars represent the mean ± s.e.m. of three independent quantitative real-time RT-PCR experiments. The *TUA1* alpha-tubulin gene (AB096718) was used as an internal control.

2.2.5 Southern hybridization

Southern hybridizations were conducted as described in (Hiwatashi et al., 2001) using a BcaBEST DNA Labeling Kit (Takara Bio Inc., Otsu, Japan). DNA fragments for the APB3ko and APB4ko probes were amplified using the PpANT4F1SalI and RPpANT12+E, and PpAP2-3 and APB3-ox-R4 primers, respectively. Probes for the *APB1-Citrine*, *APB2-GUS* and *APB3-GUS*, and *APB4-Citrine* lines were amplified using the PpAPB1-ctr-3F2 and PpAPB1-ctr-3R2, PpAP2-3-5-2 and PpANT3+HindIII, and PpAPB4-ctr-3F2 and PpAPB4-ctr-3R2 primers, respectively. A PIG1 probe for the *HSP*:*APB4* and *HSP*:*Cerulean-APB4* lines was amplified using the SSP-PIG1bRr1 and Xb-PIGRf1 primers. A pTA1 probe for *EF1-α*:*APB4* lines was amplified using the PTA1-3'f1 and PTA1-3'r1 primers.

2.2.6 Quantitative RT-PCR

A mixture of chloronemata and caulonemata was homogenized by vortexing for 1 minute using a six-well tube and ceramic balls (KURABO, Osaka, Japan), and vegetatively propagated on a BCDAT medium agar plate for four days. Then, the tissues were treated with or without phytohormones. The collected protonemata were ground in liquid nitrogen and total RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). cDNA was synthesized using the QuantiTect Reverse Transcription Kit (QIAGEN), and quantitative RT-PCR was performed using the QuantiTect SYBR Green PCR Kit (QIAGEN). The following primer pairs were used: for *APB1*, PpAPB1GSP-F1 and PpAPB1GSP-R1; for *APB2*, PpAPB2GSP-F1 and PpAPB2GSP-R1; for *APB3*, PpAPB3GSP-F1 and PpAPB3GSP-R1; for *APB4*,

PpAPB4GSP-F3 and PpAPB4GSP-R3; and for *PpTUA1*, PpTUA1F and PpTUA1R.

2.2.7 Histochemical assay for GUS activity

GUS staining was basically conducted according to Nishiyama et al. (2000). Each line was cultured on BCDAT or BCDATG medium. The tissues were not fixed before GUS staining, were infiltrated for 20 or 30 minutes in a substrate solution (50 mM NaH_2PO_4 [pH 7.0], 0.5 mM 5-bromo-4-chloro-3-indolyl ß-D-glucuronide [X-Gluc, Wako Pure Chemical Industries, Osaka, Japan], 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, and 0.05% [v/v] Triton X-100), and were then stained at 37℃ for 6-24 hours. After the incubation, the tissues were fixed in 5% (v/v) formalin for 10 minutes and then soaked in 5% (v/v) acetic acid for 10 minutes. The tissues were then dehydrated through an ethanol series. Images of the stained tissues were digitized with a charge-coupled device (CCD) camera (FUJIX HC300Z, Fuji Photo Film, Japan or CoolSNAP, Roper Scientific Photometrics, Germany).

2.2.8 Microscopy

To observe fluorescence proteins, moss was cultured on glass bottom dishes with BCDAT medium for 7 to 10 days. Digital images were obtained using a confocal microscope (A1, Nikon, Japan) with a 20×/0.75 NA objective lens. The fluorescence excitation was performed with a 514-nm Ar laser and the emission spectra were collected using a 540/30 band pass filter.

To perform time-lapse observation, moss was cultured on glass bottom dishes with BCD medium for 5 to 8 days. After cultivation, the moss plants were treated with 1/2

BCD liquid medium containing 1 μM BAP and time-lapse observation was started. Digital images were obtained using the microscope (IX81, Olympus, Japan).

2.3 Results

2.3.1 Gametophores were not formed in the *APB* quadruple disruptants

The ANT subfamily of AP2/ERF transcription factors includes *ANT*, *PLT1*, *PLT2*, *BBM*, *AIL1*, *PLT5*/*AIL5*, *PLT3*/*AIL6*, and *PLT7*/*AIL7*. These genes regulate various developmental processes including organ size determination, formation of the stem cell niche, control of phyllotaxis, and embryogenesis in *A. thaliana* (Elliott et al., 1996; Mizukami and Fischer, 2000; Boutilier et al., 2002; Hu et al., 2003; Aida et al., 2004; Galinha et al., 2007). I focused on their orthologs in *P. patens*. In addition to three reported orthologs named *PpANT1*, *PpANT2*, and *PpANT3* (Shigyo et al., 2006), I found a fourth ortholog in the genome sequence. Based on a phylogenetic analysis, the four genes belonged to same group with *ANT*, *PLT1*, *PLT2*, *BBM*, *AIL1*, *PLT5*/*AIL5*, *PLT3*/*AIL6*, and *PLT7*/*AIL7* (Fig. 6) (Kim et al., 2006; Shigyo et al., 2006; Floyd and Bowman, 2007). I therefore renamed the four *P. patens* genes *APB1* (AB675589), *APB2* (AB675590), *APB3* (AB675591), and *APB4* (AB675592), based on the initials of *ANT*, *PLT*, and *BBM*. For loss-of-function analysis of these genes, each *APB* gene was inserted or replaced with a different antibiotics resistant gene cassette by homologous recombination (Fig. 2) to form single gene disruption lines (*apb1*-7, *apb1*-119, *apb2*-55, *apb2*-61, *apb3*-30, *apb3*-32, *apb4*-103, and *apb4*-104). The protonemata and gametophores of the *apb1*, *apb2*, and *apb3* single disruption lines were not

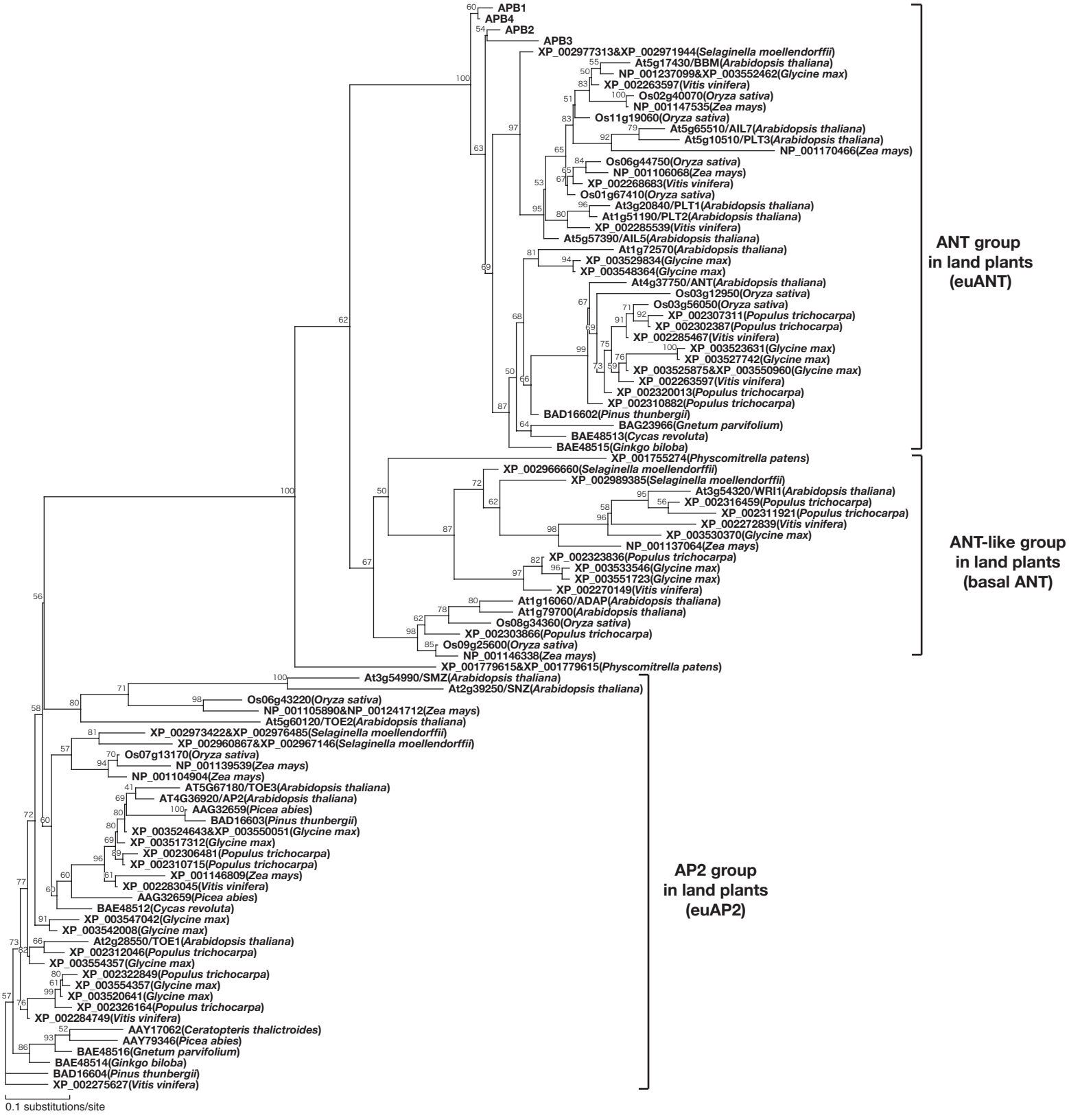

Figure 6. A maximum-likelihood tree of AP2-type transcription factors.
This tree is unrooted. Local bootstrap values are shown on branches. Horizontal branch length is proportional to the estimated evolutionary distance. Group assignments (Floyd and Bowman, 2007) are designated on the right. Genes with identical amino acid sequences within the region used for the phylogenetic analysis are combined in the same branch. Accesion numbers and species names are shown.

distinguishable from those of the wild type, but the *apb4* single disruption lines produced fewer gametophores (Fig. 7). I then constructed double (*apb2apb3*-60 and -90), triple (*apb1apb2apb3*-3 and -56), and quadruple disruption lines (*apb1apb2apb3apb4* [*apb*-quadruple]-57 and -73). The *apb2apb3* double disruption lines were indistinguishable from the wild type. On the other hand, the number of gametophores per protonema culture decreased in the *apb1apb2apb3* triple disruption lines, although the decrease was not greater than in the *apb4* single disruption lines. Finally, quadruple disruption of *APB* genes completely blocked gametophore formation. These results suggest that the four *APB* genes function redundantly in gametophore formation and that *APB4* has the major role in this process; however, it is necessary to analyze other double and triple disruption mutants to determine the precise function of each gene.

2.3.2 Protonema apical cells replace gametophore apical cells in *apb*-quadruple mutant lines

Since the *apb*-quadruple lines do not form gametophore apical cells, I next investigated whether the gametophore apical cells were replaced by secondary chloronema apical cells, secondary caulonema apical cells, or undivided cells. During the early stages of development, I could not distinguish between secondary chloronema apical cells and secondary caulonema apical cells and treated them together as secondary protonema apical cells. It is also difficult to quantify the number of secondary protonema apical cells and gametophore apical cells in regular culture conditions, because side branch formation is not synchronous. Therefore, I pre-cultured protonemata in unilateral red

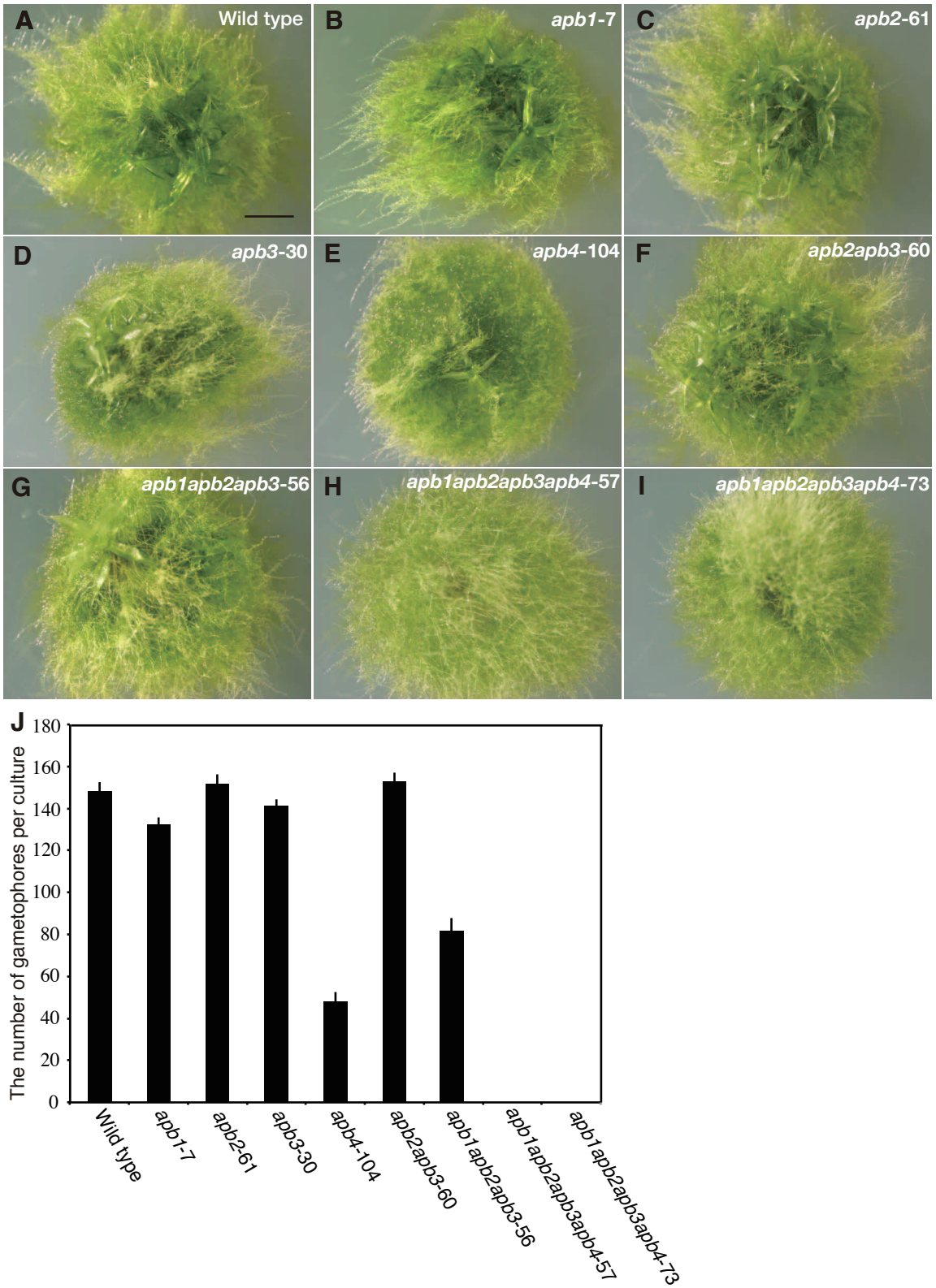

Figure 7. The number of gametophores in *APB* single, double, triple, and quadruple disruption lines.
(A-I) Protonema cultures of the wild type (A), *apb1*-7 (B), *apb2*-61 (C), *apb3*-30 (D), *apb4*-104 (E), *apb2apb3*-60 (F), *apb1apb2apb3*-56 (G), *apb1apb2apb3apb4* (*apb*-quadruple)-57 (H), and *apb*-quadruple-73 (I). A pinch of protonemata was incubated on BCDAT medium for 16 days. Scale bar: 2 mm. (J) The number of gametophores per protonema culture. Bars represent the mean ± s.e.m. derived from data of five independent colonies.

light for one week and then moved them to polarized white light (Okano et al., 2009). Under unilateral red light, caulonema cells produced almost no side branch initial cells (Fig. 8A). When caulonema filaments of both the wild type and the *apb*-quadruple lines were transferred to polarized white light and cultured for a further two days without any exogenous phytohormone, most caulonema cells synchronously underwent tip growth and formed secondary protonema apical cells. However, no gametophore apical cells formed in either the wild type or the *apb*-quadruple lines (Fig. 8B,C). When wild-type protonemata were cultured in the presence of benzylaminopurine (BAP), 5.8 ± 2.3 (s.d., n = 42) and 2.0 ± 1.7 (s.d., n = 42) of the ten caulonema cells adjacent to the caulonema apical cell gave rise to gametophore apical cells and secondary protonema apical cells, respectively (Fig. 8D and Table 2). On the other hand, no gametophore apical cells were formed and 5.4 ± 1.5 (s.d., n = 42) and 4.4 ± 1.6 (s.d., n = 43) secondary protonema apical cells were formed on the corresponding cells in the *apb*-quadruple-57 and -73 lines, respectively, upon BAP treatment (Fig. 8E and Table 2). Although the total number of side branches was slightly lower in the *apb*-quadruple lines (8.9 ± 1.5 [s.d.], n = 42 in wild type; 7.7 ± 1.4 [s.d.], n = 42 in *apb*-quadruple-57; 7.4 ± 1.9 [s.d.], n = 43 in *apb*-quadruple-73), these results indicate that gametophore apical cells were, at least partially, replaced by secondary protonema apical cells in the *apb*-quadruple lines. Whereas the number of gametophore apical cells decreased in the quadruple mutants, that of secondary protonema apical cells increased. This finding suggests that a mechanism that forms secondary protonema apical cells is activated when gametophore apical cells fail to form. The exogenous application of auxin could complement defects

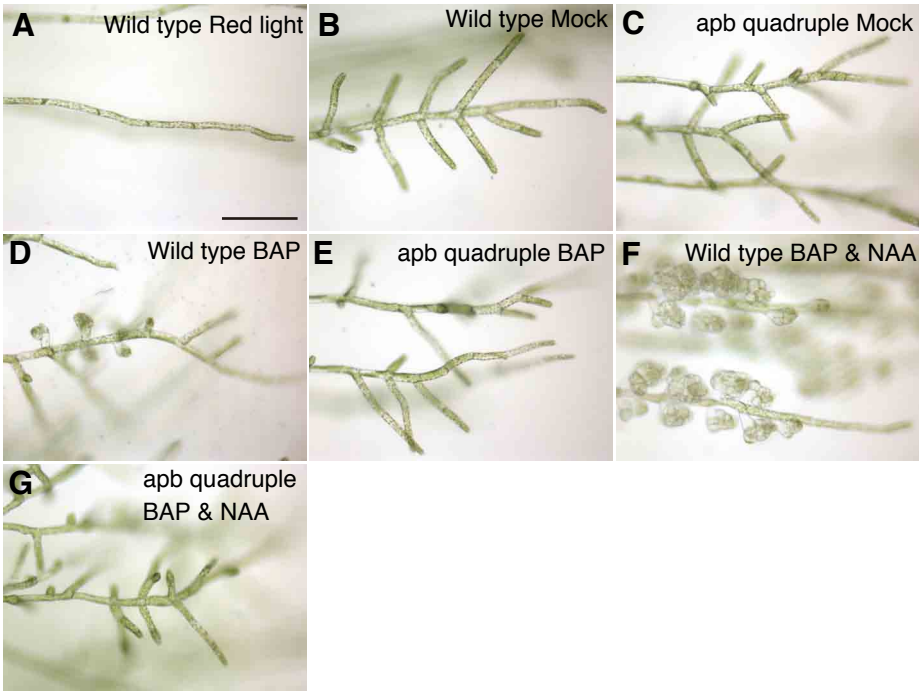

Figure 8. Gametophore apical cells are replaced by secondary protonema apical cells in *apb*-quadruple lines.
(A,B,D,F) The wild type and (C,E,G) the *apb*-quadruple-57 disruption line. Protonemata were grown in red light for one week (A) and then incubated in polarized white light for two days in the absence of BAP and NAA (B,C) or in the presence of 1 µM BAP (D,E) or 1 µM of both BAP and NAA (F,G). Scale bar: 200 µm.

Table 2. The number of gametophore apical cells and secondary chloronema apical cells in wild-type and *APB*-quadruple disruption lines

		Mock**	1 μM BAP**	1 μM BAP & 1 μM NAA**
Wild type	Gametophore apical cells	0 ± 0	5.8 ± 2.3	7.3 ± 1.8
	Secondary protonema apical cells	5.2 ± 1.3	2.0 ± 1.7	0.2 ± 0.5
	Unidentified side branch initial cells*	1.1 ± 1.1	1.1 ± 1.1	0.4 ± 1.2
	Side branches in total	6.4 ± 1.4 (n = 55)	8.9 ± 1.5 (n = 42)	7.9 ± 1.4 (n = 54)
apb1apb2apb3apb4-57	Gametophore apical cells	0 ± 0	0 ± 0	0.2 ± 0.5
	Secondary protonema apical cells	5.1 ± 1.5	5.4 ± 1.5	2.5 ± 1.1
	Unidentified side branch initial cells*	1.4 ± 1.1	2.2 ± 1.3	3.6 ± 1.6
	Side branches in total	6.5 ± 1.4 (n = 48)	7.7 ± 1.4 (n = 42)	6.3 ± 2.0 (n = 51)
apb1apb2apb3apb4-73	Gametophore apical cells	0 ± 0	0 ± 0	0.1 ± 0.3
	Secondary protonema apical cells	4.9 ± 1.6	4.4 ± 1.6	2.2 ± 1.6
	Unidentified side branch initial cells*	0.9 ± 1.0	3.0 ± 1.9	4.1 ± 2.1
	Side branches in total	5.8 ± 1.8 (n = 50)	7.4 ± 1.9 (n = 43)	6.4 ± 2.3 (n = 40)

Protonemata were cultured under red light for one week, and then cultured under polarized white light for two days.
The number of gametophore apical cells, secondary protonema apical cells, unidentified side branch initial cells, and side branches in total that emerged from ten caulonema cells next to caulonema apical cells, were counted.

* The number of side branch initial cells whose identity can not be defined as a gametophore apical cell or a secondary chloronema apical cell.

** The values indicate the mean with standard deviation.

in gametophore formation in some cytokinin-resistant mutants (Ashton et al., 1979). However, the exogenous application of both cytokinin and auxin did not induce gametophore apical cells in the *apb*-quadruple-57 and -73 lines (Fig. 8F,G) with few exceptions (Table 2).

Although side branch initial cells differentiate into protonema apical cells in the *apb*-quadruple lines, such side branch initial cells might have gametophore apical cell identity transiently. Therefore, time-lapse observation of secondary apical cell formation process was performed (Fig. 9 and Table 3). It seems that at least based on their morphology, side branch initial cells do not acquire gametophore apical cell identity and directly differentiate into protonema apical cells.

2.3.3 *APB* genes were continuously expressed during gametophore apical cell formation

To examine the functions of the *APB* genes, I analyzed the expression patterns of the APB proteins during the formation of secondary protonema apical cells and gametophore apical cells. The *Citrine* yellow fluorescent protein gene (Heikal et al., 2000) or the *uidA* gene, which encodes ß-glucuronidase (GUS) (Jefferson, 1987), was inserted just before the stop codon of each *APB* gene by means of homologous recombination. I selected single insert lines, determined by Southern hybridization, for further analysis (Fig. 3). All four fusion proteins localized to caulonema cells before the formation of apical cells (Fig. 10A,F,K,P,U,Z). A side branch initial cell is initiated as a protrusion at the surface of caulonema cells and divides from the parental caulonema cell. Citrine and GUS signals were detected in both the side branch initial and parental

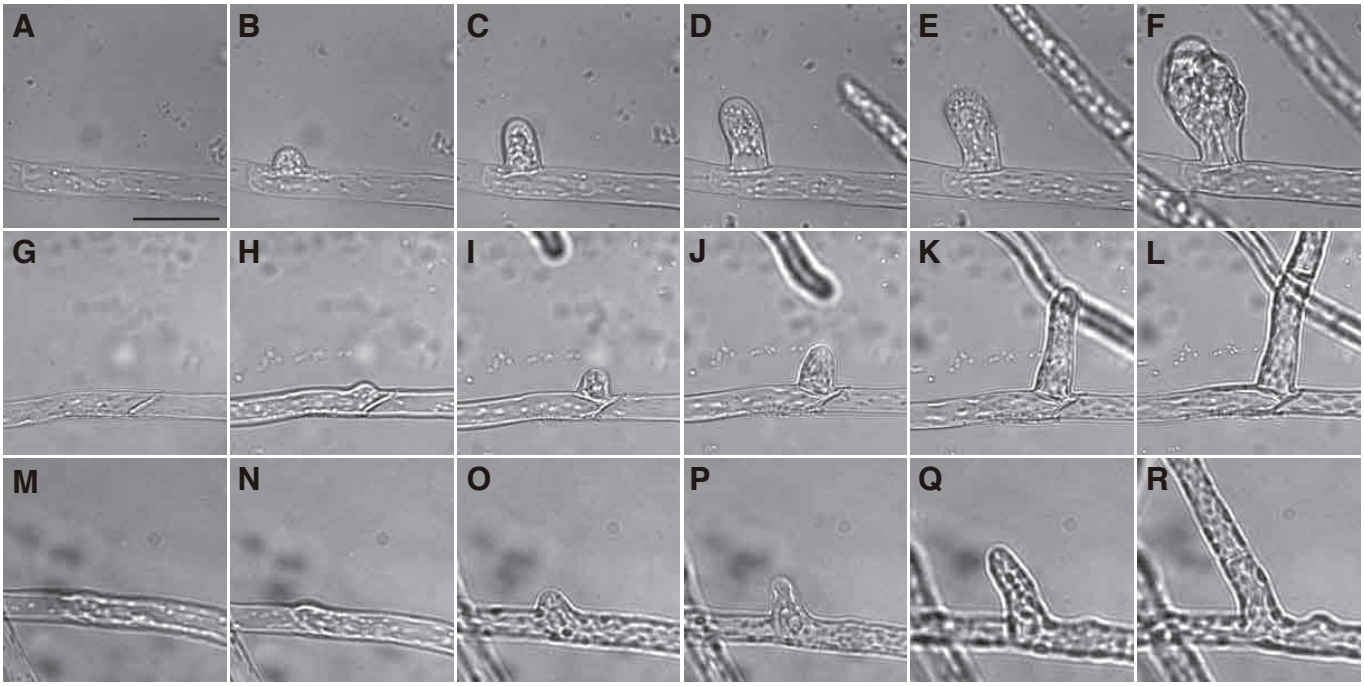

Figure 9. Time-lapse observation of apical cell formation in wild-type and *APB*-quadruple lines.
(A-R) Composited of bright field images of wild-type (A-F) and *apb*-quadruple-57 (G-L) and *apb*-quadruple-73 (M-R) during the formation of gametophore or protonema apical cells 0 hour (A, G, and M), 3 hours (B, H, and N), 9 hours 40 minutes (C, I, and O), 13 hours (D, J, and P), 16 hours 20 minutes (E, K, and Q), and 33 hours (F, L, and R) after starting observation. Scale bars: 50 μm.

Table 3. The percentage of gametophore apical cells and secondary chloronema apical cells in time-lapse observation

	Wild type (n = 110)	*apb1apb2apb3apb4*-57 (n = 115)	*apb1apb2apb3apb4*-73 (n = 84)
Gametophore apical cells	98 %	1 %	1 %
Secondary chloronema apical cells	2 %	99 %	99 %

Protonemata were cultured on BCD medium under white light for 5-8 days, and then treated with 1/2 BCD liquid meidum containing 1 μM BAP for 36 hours.

The percentage of gametophore apical cells and secondary chloronema apical cells, which differentiated from newly formed side branch initial cells, were caluculated.

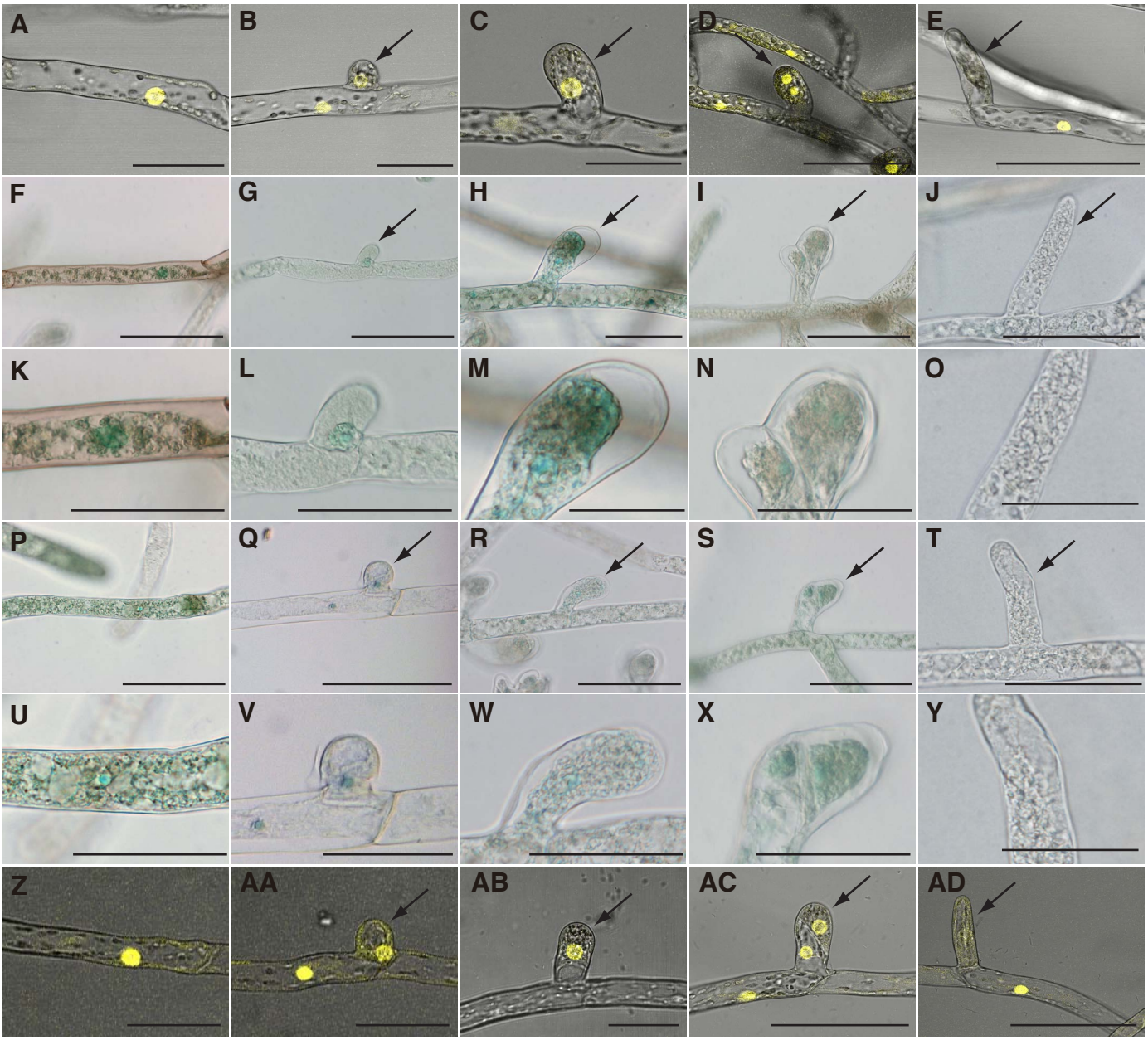

Figure 10. APB-reporter fusion proteins were detected during gametophore apical cell dormation but not during secondary protonema apical cell formation.
(A-AD) Composites of bright field images and fluorescence images of APB1-Citrine (A-E) and APB4-Citrine (Z-AD) lines and bright field images of APB2-GUS (F-O) and APB3-GUS (P-Y) lines in a caulonema cell before the initiation of an apical cell (A,F,K,P,U,Z), in a protruded side branch initial cell (arrows) and a parental caulonema cell just after cell division (B,G,L,Q,V,AA), in a swollen gametophore apical cell (C,H,M,R,W,AB; arrow), in a gametophore apival cell (arrows) and its daughter cell (D,I,N,S,X,AC), and in a secondary protonema apical cell (arrows in E,J,O,T,Y,AD). Magnified pictures of F,G,H,I,J,P,Q,R,S, and T are shown in K,L,M,N,O,U,V,W,X, and Y, respectively.
Scale bars: 50 µm (A-C,K-O,U-AB), 100 µm (D-J,P-T,AC,AD).

caulonema cells in the *APB1-Citrine*, *APB3-GUS*, and *APB4-Citrine* lines (Fig. 10B,Q,V,AA), whereas signals were detected only in the side branch initial cells in the *APB2-GUS* lines (Fig. 10G,L). At this stage, the morphology of a side branch initial cell did not reveal whether the cell was fated to become a gametophore or a secondary protonema apical cell. Subsequently, approximately 5% of side branch initial cells swelled up and divided obliquely to form a gametophore apical cell, while the remaining 95% of side branch initial cells continued to undergo tip growth without swelling and became secondary protonema cells (Fig. 1). The APB-Citrine and APB-GUS signals were detected in swollen cells (Fig. 10C,H,M,R,W,AB, and Table 4). I detected APB-reporter signals of all *APB* genes in the gametophore apical cell and in the gametophore cells derived from the gametophore apical cell (Fig. 10D,I,N,S,X,AC). On the other hand, APB-reporter signals were not usually detected in apical cells undergoing tip growth to become secondary protonema apical cells (Fig. 10E,J,O,T,Y,AD), and signals were occasionally observed in secondary protonema apical cells in each reporter line. To quantitatively examine the expression patterns, I further analyzed *APB1-Citrine* and *APB4-Citrine* lines, because the *apb4* single deletion mutant showed the strongest phenotype and *APB1* is sister to *APB4* (Table 4). APB-Citrine signals were detected in all mother protonema cells and more than 85% side branch initial cells. More than 70% side branch initial cells with swelling showed Citrine signals, while less than 15% side branch initial cells with secondary protonema characters showed signals.

Table 4. The percentage of side branch initial cells with APB-Citrine signal

	APB1-Citrine	APB4-Citrine
The third cells from apical cells	100 % (n = 24)	100 % (n = 35)
Cells just after cell division*	96 % (n = 26)	85 % (n = 40)
Cells fated to secondary protonema apicel cells*	11 % (n = 19)	13 % (n = 23)
Cells fated to gametophore apical cells with swelling*	92 % (n = 12)	73 % (n = 11)

* Side branch initial cells with more than three times fluorescence intensity than their mother cells were counted.

2.3.4 Induction of *APB4* increased the number of gametophores

To further examine the roles of APB in the regulation of gametophore apical cell formation, I generated heat shock-inducible transgenic lines of *APB4*, which has the most important function of the four *APB* genes (Fig. 4). In addition to lines containing *APB4* fused to the soybean *Gmhsp17.3B* heat-shock promoter (Saidi et al., 2005) (*HSP:APB4*-12 and *HSP:APB4*-120 lines), transgenic lines containing *APB4* fused to a *Cerulean* cyan fluorescent protein gene, *Cerulean* (Rizzo et al., 2004), and HSP (*HSP:Cerulean-APB4*-264 and *HSP:Cerulean-APB4*-284 lines) were generated to monitor induction of the gene (Fig. 4). Transgenic lines containing *Cerulean* fused with HSP were also generated as a control (*HSP:Cerulean*-2 line). The amount of *APB4* mRNA increased in *HSP*:*APB4* and *HSP*:*Cerulean-APB4* lines upon heat shock induction, but did not increase in the *HSP:Cerulean* line (Fig. 11A). *APB4* transcripts were more strongly induced in *HSP*:*Cerulean-APB4* lines than in *HSP*:*APB4* lines, for unknown reasons. The Cerulean signal of *HSP*:*Cerulean-APB4* lines was detected in all protonema cells exposed to heat shock (Fig. 4). In the absence of induction, the *APB4* inducible lines were indistinguishable from the wild type and formed a similar number of gametophores (Fig. 11B). In contrast, the number of gametophores increased in *HSP*:*APB4* and *HSP*:*Cerulean-APB4* lines upon induction, but not in *HSP:Cerulean* lines (Fig. 11B). Although the number of gametophores increased, other side branch initial cells of *HSP*:*APB4* and *HSP*:*Cerulean-APB4* lines changed to secondary protonema apical cells.

To investigate the proportion of gametophore apical cells to secondary

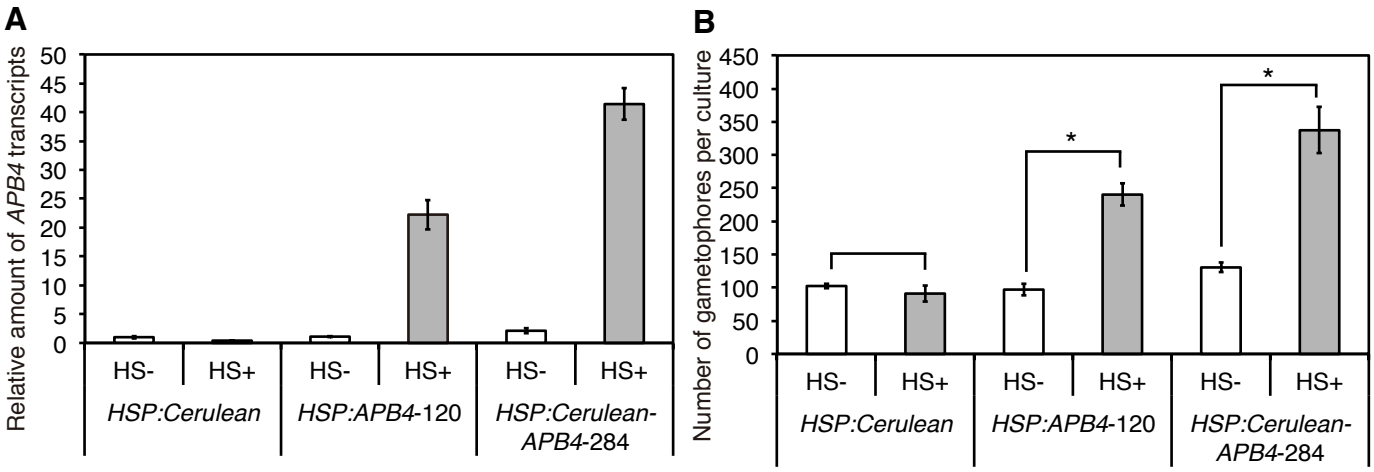

Figure 11. Induction of APB4 transcripts increased the number of gametophores.
(**A**) Quantitative real-time RT-PCR analysis of *APB4* transcripts in transgenic lines subjected or not to heat-shock induction. Protonemata were collected after cultivation at 38°C for 1 hour. Error bars represent the mean ± s.e.m. of theree independent quantitative real-time RT-PCR experiments. The *TUA1* alpha-tubulin gene (AB096718) was used as an internal control. (**B**) The number of gametophores per protonema culture grown in white light for two weeks with or without heat-shock at 38°C for 1 hour of every 12 hours. Bars represent the mean ± s.e.m. of data derived from five independent cultures. * indicates a significant difference relative to non-heat-shock conditions ($p<0.01$ t-test).

chloronema apical cells with *APB4* induction, I cultivated *HSP:Cerulean-APB4* lines under the red light conditions. However, side branch formation was arrested with heat-shock under the red light conditions because of unknown reason. I therefore generated constitutive over-expression lines of *APB4* (Fig. 5). When protonemata were treated with 0.1 μM BAP, 2.7 ± 2.0 (s.d., n = 36) gametophore apical cells in the ten caulonema cells adjacent to the caulonema apical cell were formed in wild type, while the number increased in *EF1-α:APB4*-82 and *EF1-α:APB4*-259 lines (3.8 ± 1.6 [s.d., n = 44] and 4.9 ± 1.6 [s.d., n = 57], respectively) (Table 5).

The over-expression of *APB4* gene also affects caulonema differentiation. The formation of caulonemata was observed within two weeks in wild type and *HSP:APB4* lines without heat shock (Fig. 12A,E). However, caulonema formation of *HSP:APB4* lines was arrested with heat shock (Fig. 12B-D,F-H). The formation of caulonemata is positively regulated by auxin (Cove et al. 2006). The defect in caulonema formation in the over-expression line was complemented by the addition of NAA to the medium (Fig. 12I,J).

2.3.5 Auxin induces the expression of *APB* genes, but cytokinin functions in parallel with auxin and APBs

I have demonstrated that *APB*s are indispensable for the formation of gametophore apical cells and that the overexpression of *APB4* enhances the formation of gametophore apical cells. The formation of gametophore apical cells is known to be regulated by the phytohormones auxin and cytokinin (Ashton et al., 1979; Cove et al., 2006). I thus analyzed the regulation of *APB* transcripts by these phytohormones using

Table 5. The number of gametophore apical cells and secondary chloronema apical cells in wild-type and *EF1-α:APB4* lines

		Wild type**	*EF1-α:APB4*-82**	*EF1-α:APB4*-259**
0.1 μM BAP	Gametophore apical cells	2.7 ± 2.0	3.8 ± 1.6	4.9 ± 1.6
	Secondary protonema apical cells	4.7 ± 2.1	3.9 ± 1.6	1.7 ± 1.1
	Unidentified side branch initial cells*	1.1 ± 1.1	0.6 ± 0.8	0.7 ± 0.8
	Side branches in total	8.5 ± 1.4 (n = 36)	8.3 ± 1.3 (n = 44)	7.4 ± 1.4 (n = 57)

Protonemata were cultured under red light for one week, and then cultured under polarized white light for two days.
The number of gametophore apical cells, secondary protonema apical cells, unidentified side branch initial cells, and side branches in total that emerged from ten caulonema cells next to caulonema apical cells, were counted.

* The number of side branch initial cells whose identity can not be defined as a gametophore apical cell or a secondary chloronema apical cell.

** The values indicate the mean with standard deviation.

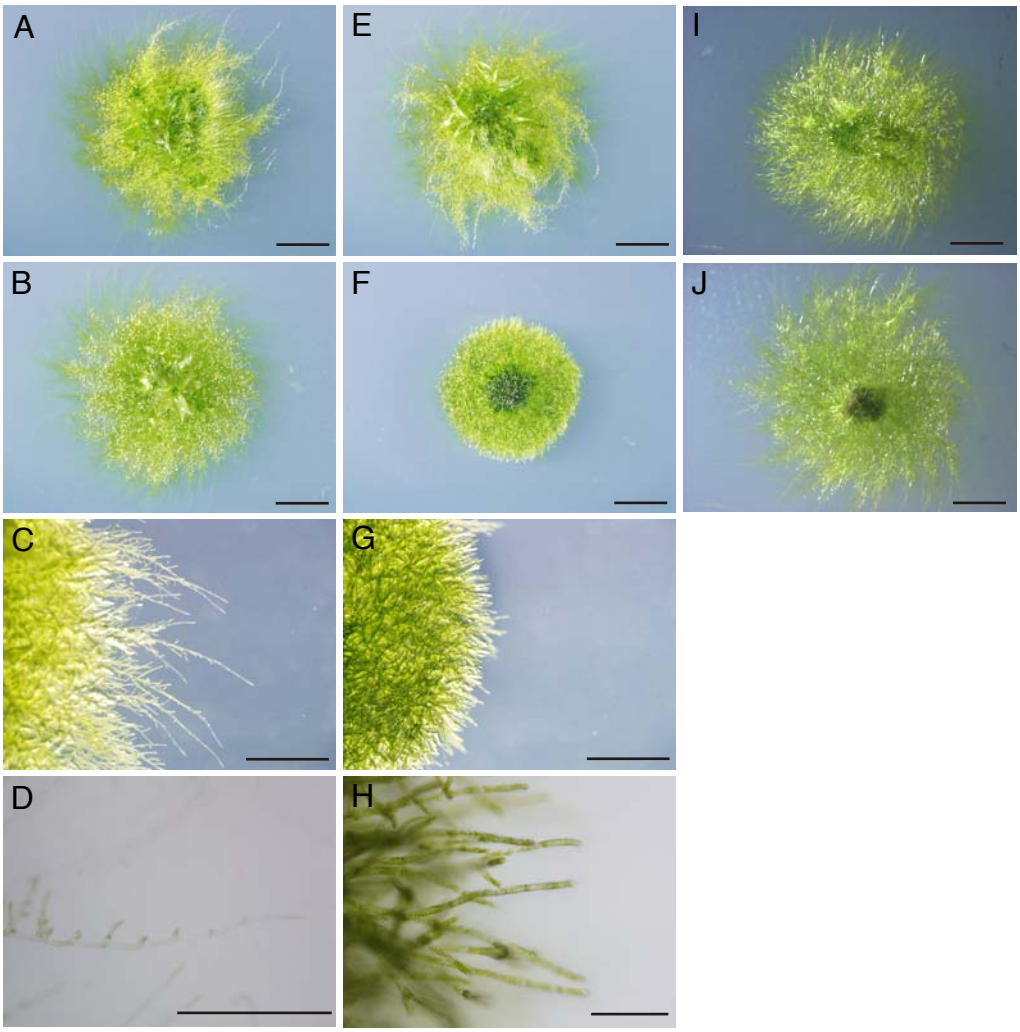

Figure 12. Induction of *APB4* diminishes caulonema formation.
(**A-J**) Protonema cultures of *HSP:Cerulean-2* (A-D,J) and *HSP:APB4-120* (E-I) lines were cultivated under white light for two weeks in the absence (A,E) or presence (B-D,F-I) of periodic heat shock treatments, showing arrest of caulonema formation in the mutant (F-H). (D,H) Magnified pictures of protonemata at the edge of the culture. (I,J) Arrest of caulonema formation was complemented by the addition of exogenous auxin (1 µM NAA). Scale bars: 2 mm (A,B,E,F,I,J), 1 mm (C,G), 200 µm (D,H).

quantitative real-time RT-PCR (Fig. 13). The accumulation of each *APB* transcript significantly increased after auxin application, whereas cytokinin application did not have a significant effect on the accumulation. The amount of *APB1*, *APB3*, and *APB4* transcript was approximately five-fold higher 12 hours after the addition of auxin than in the absence of auxin (Fig. 13A,C,D). *APB2* transcript levels were strongly induced (by up to approximately 15-fold) by auxin (Fig. 13B). Small synergistic effects of cytokinin and auxin were observed. *APB2* expression is enhanced by the combined addition of auxin and cytokinin while cytokinin slightly suppresses the auxin-induction of *APB3* (Fig. 13). These results indicate that all four *APB* genes are positively regulated by auxin, but not by cytokinin. I also found that expression patters were not changed by the phytohormones in both *APB1-Citrine* and *APB4-Citrine* lines (Fig. 14).

2.4 Discussion

2.4.1 *APB* genes are master regulators of gametophore apical cell formation in *P. patens*

This study shows that disruption of all of four *P. patens* AP2-type transcription factor *APB*s caused defects in the formation of gametophore apical cells (Fig. 7) and promoted the formation of secondary protonema apical cells as compensation (Fig. 8). An APB-reporter protein fusion was detected in gametophore apical cells, but was largely absent from secondary protonema apical cells (Fig. 10). *APB* expression in side branch initial cells continues during gametophore apical cell formation, but disappears when secondary protonema cells are formed. This suggests that continuous expression of *APB*s is required for gametophore apical cell formation. Future experiments to

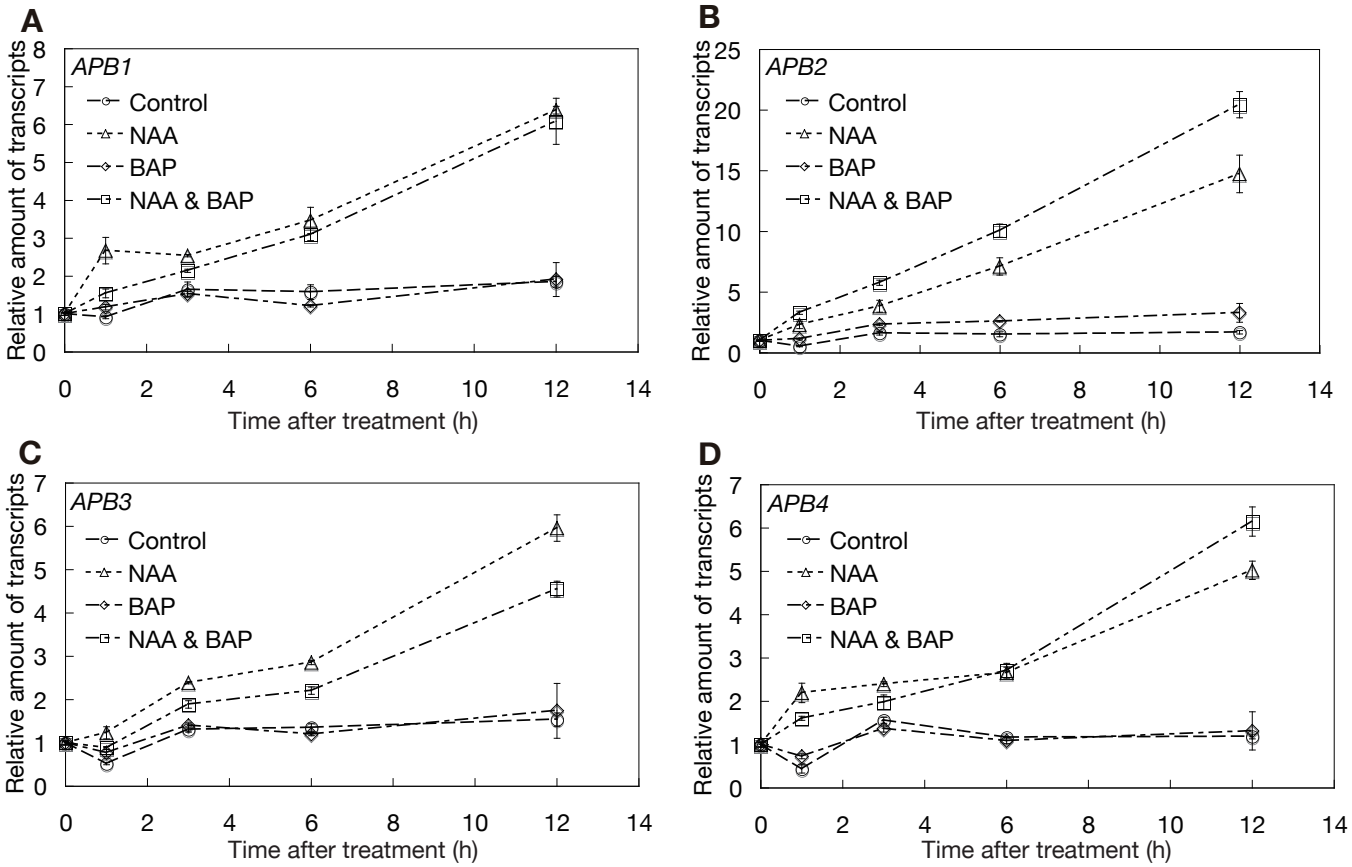

Figure 13. Auxin induces *APB*, wheres cytokinin does not.
(**A-D**) Relative transcript levels of *APB1* (A), *APB2* (B), *APB3* (C), and *APB4* (D), as determined by quantitative real-time RT-PCR, in wild-type moss cultured in mock solution and in the absence of exogenously applied phytohormones (circle), with 1 μM NAA (triangle), 1 μM BAP (diamond), or 1 μM of both NAA and BAP (square). Bars represent the mean ± s.e.m. of three independent quantitative real-time RT-PCR experiments. The *TUA1* alpha-tubulin gene was used as an internal control. The value at 0 hour was taken as 1.0.

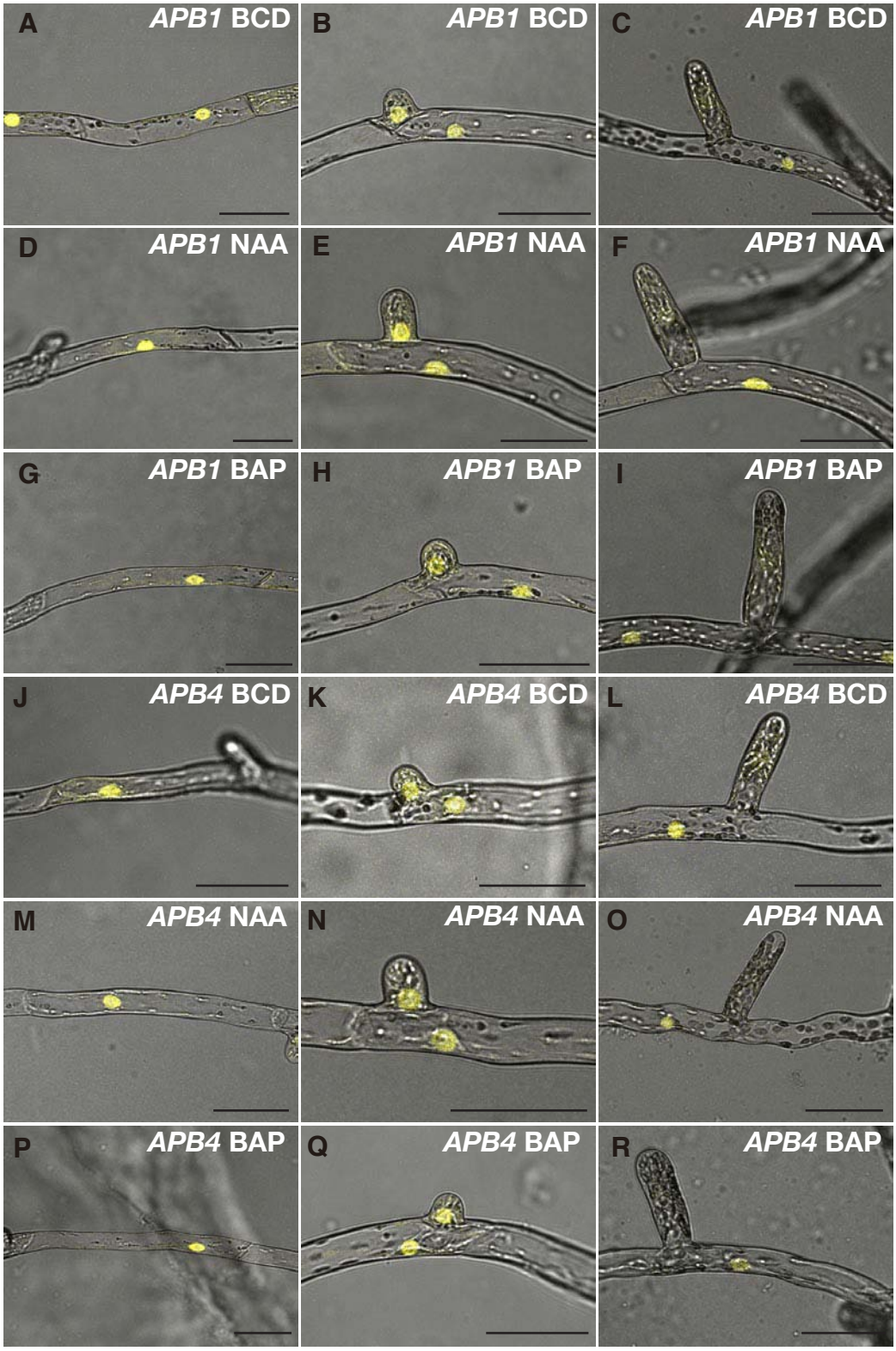

Figure 14. Effects of phytohormone on expression of APB4-Citrine signal.
(**A-R**) Composites of bright field images and fluoresncence images of APB1-Citrine (A-I) and APB4-Citrine (J-R) fusion proteins in a caulonema cell before the initiation of an apical cell (A,D,G,J,M,P), in a protruded side branch initial cell (B,E,H,K,N,Q), and in a secondary protonema apical cell (C,F,I,L,O,R). Protonema cells were treated without (A-C,J-L), or with 1 µM NAA (D-F,M-O) and 1 µM BAP (G-I,P-R). Scale bars: 50 µm.

manipulate *APB* expression levels during each apical cell formation will be useful to examine this hypothesis and to reveal the timing of fate decision (Fig. 10 and Table 4). Overexpression of *APB4* increased the proportion of gametophore apical cells to secondary protonema apical cells (Fig. 11 and Table 5), although secondary protonema apical cells were still formed. Together, these results suggest that *APB*s are indispensable but not sufficient molecular switches in the formation of gametophore apical cells. *APB*-quadruple lines produced a few gametophore apical cells with the addition of exogenous cytokinin and auxin (Table 2), suggesting other redundant genes or pathways.

2.4.2 Interaction of *APB*s with auxin and cytokinin

Gametophores are induced by exogenously applied cytokinin (Cove et al. 2006). Previously isolated *P. patens* mutants, BAR, PC22, and P24, exhibited a reduced number of gametophores in comparison to the wild type, although the genes responsible have not been identified. This defect was complemented by the exogenous addition of cytokinin or the induction of a cytokinin biosynthesis gene (Ashton et al., 1979; Abel et al., 1989; Reutter et al., 1998). In addition, the number of gametophores decreased in the BAR 77 mutant line, and the exogenous addition of auxin complemented the defect, indicating that auxin is also involved in the formation of gametophore apical cells (Ashton et al. 1979). This study showed that auxin positively regulates the expression of *APB* genes (Fig. 13) and that *APB*s are necessary for the cytokinin signaling-mediated formation of gametophore apical cells (Fig. 8). The regulation of *APB*s is reminiscent of

the auxin-mediated regulation of *ANT* via ARGOS (Hu et al., 2003) and *PLT*s (Aida et al., 2004; Galinha et al., 2007). As the auxin perception pathway using TIR1 and Aux/IAA in *A. thaliana* is conserved in *P. patens* (Prigge et al., 2010), the regulatory gene networks of the ANT subfamily may also be conserved between these two distantly related plants. However, I could not find *ARGOS* orthologs in the *P. patens* genome (Banks et al., 2011), and the network is therefore expected to be partly different.

Since proteins in the AP2 family function as transcription factors and *APB*s are necessary for cytokinin signaling, APB proteins may regulate the expression of cytokinin signaling genes, such as response regulators. Alternatively, given the role of the AP2 domain in protein-protein interactions (Chandler et al., 2007; Chandler et al., 2009; Lee et al., 2010), APB proteins may interact with proteins that function in cytokinin signaling. Indeed, it was recently demonstrated that AP2-type transcription factors form heterodimers that control embryogenesis or the stress response (Chandler et al., 2007; Chandler et al., 2009; Lee et al., 2010). Similar interactions between APB and other factors may regulate stem cell formation in *P. patens*. Future isolation of the targets and factors that interact with APB will provide insight into the role of APB in cytokinin signaling.

2.4.3 APBs are candidate targets of local cues for fate determination

Since gametophore apical cells and secondary protonema apical cells are sometimes observed next to each other on the same caulonema cell, it is speculated that

intracellular cues exist in a parent caulonema cell that determine the fate of each gametophore apical cell and secondary protonema apical cell (Harrison et al., 2009). The authors discussed the involvement of a local cytokinin gradient and auxin transport to a restricted region in establishing the local cue that determines the fate of side branch initial cells. An APB-reporter fusion protein was broadly expressed in caulonema cells and in side branch initial cells, which become either gametophore apical cells or secondary protonema apical cells (Fig. 10). The fusion protein disappeared in secondary protonema apical cells upon the initiation of tip growth, but was continuously detected in gametophyte apical cells, which swell but do not undergo further tip growth. Incorporating the auxin-mediated regulation of *APB* (Fig. 10), a local loss of auxin in presumptive protonema apical cells may serve as a cue during the cell fate determination. To elucidate this unique developmental mechanism that relies on local cues, it is necessary to determine the local distribution of active auxin, the localization of auxin transporters, the expression of genes involved in auxin metabolism, and the effect of cytokinin signaling.

2.4.4 The function of APBs in other developmental processes

Although this study focused on the formation of gametophore and secondary protonema apical cells, in addition to the increase in gametophores, I observed several other phenotypes in the *APB4* overexpression lines, such as partial defects in caulonema formation (Fig. 12). Caulonema formation is regulated by auxin and the defect in these lines was complemented by the addition of auxin (Fig. 12I). This finding implies that

endogenous auxin levels in the overexpression lines are lower than those in the wild type and raises the possibility that *APB4* negatively regulates auxin biosynthesis. Since the accumulation of *APB4* transcripts was up-regulated by auxin (Fig. 13), there may be a negative feedback loop between auxin biosynthesis and *APB* genes.

3. GENERAL DISCUSSION

3.1 Common molecular mechanisms between stem cells in gametophyte and sporophyte generation

In addition to the characterization of a transcription factor that functions in the gametophyte generation, this study implicated a potential similarity of molecular mechanisms of stem cell formation in *P. patens* gametophytes and *A. thaliana* sporophytes. Class III HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIP III) genes and *PLT* genes are respectively master regulators of shoot and root with mutually antagonistic transcriptional regulation (Smith and Long, 2010), although it is still unclear these genes are involved in not only shoot and root specification but also shoot and root apical meristem specification. *P. patens* orthologs to *A. thaliana* HD-ZIPIII genes exist in its genome (Banks et al., 2011) but functions of any genes have not been reported. Future characterization of these genes in both species may show similar genetic circuits in fate decision of stem cells with different characters.

ANT, *AIL6*, and *AIL7* regulate shoot apical meristem function in *A. thaliana* (Mudunkothge and Krizek, 2012). These studies suggest that genes in the ANT subfamily are widely involved in stem cell regulation in the sporophyte generation in *A. thaliana*. My doctoral thesis showed that genes in the ANT subfamily are important for stem cell regulation in the gametophyte generation in *P. patens*. Therefore, it is likely that there are the common molecular mechanisms for stem cell regulation between gametophyte and sporophyte generations, in which genes in ANT subfamily play a critical role.

Previous studies demonstrated that orthologs of Class 1 *KNOX* genes regulate

sporophyte development and orthologs of *WOX* genes regulate regeneration from leaf cells and sporophyte development in *P. patens* (Sakakibara et al., 2008; Sakakibara et al., submitted). Orthologs of these genes in *A. thaliana* are involved in the stem cell regulation and development of a sporophyte (Endrizzi et al., 1996; Mayer et al., 1998; Schoof et al., 2000). These results suggest the possibility that molecular mechanisms for stem cell regulation in the sporophyte generation evolved using genes, which function in the sporophyte generation of ancestral organism. This hypothesis conflicts with "co-option" hypothesis. However, in this study, I showed that *APB* genes in ANT subfamily regulate the formation of gametophore apical stem cells in *P. patens*. Orhologs of *APB* genes regulate development in the sporophyte generation including stem cell niche formation, formation of a SAM, control of phyllotaxis, cell proliferation, and organ growth in *A. thaliana* (Aida et al., 2004; Nole-Wilson et al., 2005; Prasad et al., 2011; Mudunkothge and Krizek, 2012). In contrast to the hypothesis discussed above, these results are consistent with the "co-option" hypothesis. Therefore, it seems to be reasonable to consider that the developmental mechanisms in the sporophyte generation were co-opted partly from pre-existing mechanisms in the gametophyte generation and also utilized genes functioned in the sporophyte generation of ancestral organisms during evolution of land plants.

3.2 Future perspective

The present study provided a new insight into the regulation of stem cell characters in the gametophyte generation. The *APB* genes work under auxin signaling and function

synergistically with cytokinin signaling to determine the identity of the two types of stem cells. However, it seems that *APB*s are indispensable but not sufficient for the formation of gametophore stem cells, because overexpression of *APB4* slightly increased the proportion of gametophore apical cells to secondary protonema apical cells, and many secondary protonema apical cells were still formed. In contrast, application of cytokinin, which did not induce the expression of *APB* genes, is enough to differentiate all side branch initial cells into gametophore apical cells. Therefore, revealing how *APB* genes interact with cytokinin signaling is a next step to elucidate molecular mechanisms for stem cell regulation in the gametophyte generation. For such a purpose, high throughput sequencers (e.g. SOLiD and PacBio) are useful to identify downstream genes of *APB* genes. Such sequencers enable us to acquire a huge amount of small sequences of expressed genes in each cDNA libraries and it is possible to reveal the expression profile by comparing the sequence data with the genome. I am trying to analyze the interaction mechanisms between *APB*s and cytokinin signaling using this method.

Although a reverse genetics is a powerful tool to analyze particular genes, a forward genetics is also a useful tool to isolate genes involved in phenomena of interest. Defects in stem cells sometimes disrupt their life cycle. In such a case, it is not possible to perform map-based cloning. Actually, *APB* quadruple disruptant lines failed to form gametophores, which are necessary to produce sexual organs. Therefore, a system that can isolate a gene directly is needed for the application of a forward genetic approach to the study of stem cell regulation. Tagged mutants were previously generated by shuttle

mutagenesis in *P. patens* (Nishiyama et al. 2000), but most of the mutants contained several tags, which made it difficult to isolate the gene responsible for the mutant phenotype. A method for detecting deletions using a genomic tiling array is better suited to identifying the affected genes in deletion mutants. I created a genomic tiling array for *P. patens* that covers the entire genome (approximately 480 Mb) at a 67-bp resolution, excluding repeated sequences. I am generating deletion mutant lines and identifying the deleted regions by hybridization of the genomic tiling array with genomic DNA. *Agrobacterium tumefaciens*-mediated transformation is a common forward genetic approach in angiosperm; in addition, this method can also be used to identify a responsible gene using the T-DNA as a tag. This approach is now being established in *P. patens*. Both reverse and forward genetic approaches will provide a new insight into regulation of stem cells in gametophyte generation.

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